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PRINCIPAL INVESTIGATOR: Allan Bradley, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
                            Houston, Texas   77030

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Allan Bradley, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, Texas 77030

E-Mail: abradley@bcm.tmc.edu

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Breast cancer is a genetic disease involving both gain and loss of function mutations in many different genes. It is important to define which genes are significant mutational targets in sporadic breast tumors so that treatments can be directed based on the knowledge of the genetic changes in the tumor. This project is focused on identifying tumor suppressor genes, which are mutated in sporadic breast cancer using a novel genetic screen. This genetic screen involves applying chromosome engineering technology to delete specific chromosomal regions in mouse mammary epithelial cells *in vivo*, which should allow the induction of tumor suppressor mutations in these haploid regions. We have established that tissue specific expression of cre can recombine *loxP* sites with 10% efficiency even when they are several Megabases apart. We used an existing transgenic cre strain, which is expressed in the heart. In order to achieve recombination in mammary epithelial cells we have been developing other cre lines. These include an *MMTV-Cre* line targeted to the *Hprt* locus as well as a line in which Cre has been knocked into the beta-casein locus. We have been evaluating the specificity of these lines using a reporter strain in which *lacZ* is activated following the expression of Cre. When a line with the desired tissue specificity is obtained, tissue specific induced chromosome deletions will be performed with relevant chromosomal regions. Substantial progress on this work was published (Methods 24:81-94, 2001).

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Allan Bradley, PhD

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Final Report for Award Number DAMD17-98-1-8280

Abstract

Breast cancer is a genetic disease and it is important to define which genes are significant mutational targets in sporadic breast tumors so that treatments can be directed based on the knowledge of the genetic changes in the tumor. This project was focused on identifying tumor suppressor genes which are mutated in sporadic breast cancer using a novel genetic screen. This genetic screen involved applying chromosome engineering technology to delete specific chromosomal regions in mouse mammary epithelial cells *in vivo*, which should allow the induction of tumor suppressor mutations in these haploid regions. We have established that tissue specific expression of cre can efficiently recombine *loxP* sites even when they are several megabases apart. In order to achieve recombination in mammary epithelial cells we have been developing several cre lines. These include an *MMTV-Cre* line targeted to the *Hprt* locus as well as a line in which Cre has been knocked into the beta-casein locus. We have been evaluating the specificity of these lines using a reporter strain in which *lacZ* is activated following the expression of Cre. When a line with the desired tissue specificity is obtained, tissue specific induced chromosome deletions will be performed with relevant chromosomal regions. Substantial progress on this work was published (Methods 24: 81-94, 2001)

INTRODUCTION

This project was focused on identifying tumor suppressor genes which are mutated in sporadic breast cancer. We proposed to try and identify these tumor suppressor genes by a genetic screen *in vivo* which involved adapting our chromosome engineering technology to delete specific chromosomal regions in mouse mammary epithelial cells in mice. We several developed lines of mice which exhibit tissue specific expression of Cre in mammary epithelial cells and attempted to use these lines to induce long-range deletion events *in vivo*. Tissue specific induced chromosome deletion *in vivo*, when combined with insertional mutagenesis has the power to directly identify tumor suppressor genes mutated in sporadic breast cancer.

(6) BODY

A. Demonstration of long-range recombination both in embryonic stem cells *in vitro* and in specific tissues *in vivo* (Zheng et al., Methods, 24: 81-94, 2001 and previous publications).

B. Demonstration of very long range recombination in ES cells allowing us to generate inversion chromosomes for genetic screens (Yu et al., (2001), Zheng et al., (2001) and previous publications). This extension of the original goal has been pursued because recombination over large distances was much more efficient than expected. We have now used this strategy to generate inversions over 10% of the mouse genome which includes all of chromosome 11 and most of chromosome 4.

C. Generation and evaluation of several mammary specific cre-alleles and one ubiquitous allele. Summary of available mouse lines:

(i) *ß–casein knock-in* . This line was generated by a knock-in of *cre* into the beta-casein locus. The cre cassette is activated by the endogenous *ß–casein* promoter followed by splicing from *ß–casein* to Cre. Although our initial assessment of this transgene is that it worked very well, (since mammary glands were stained entirely blue by X-gal in crosses to ROSA26-reporter mice), upon further investigation we discovered that recombination appeared to occur during early embryonic development. This became obvious when we performed lacZ staining of embryos. In this case we detected X-gal staining in many different tissues. This "ectopic" expression of cre resulted in recombination in multiple tissues, which would prevent this allele being used for our intended purpose to just induce recombination in mammary epithelial cells, because of developmental effects caused by large regions of hemizygosity in many different tissues. Attempts to retrofit this allele were not successful.

(ii) *Hprt–MMTV-cre allele*. This allele is a multiple copy insertion of an MMTV-cre transgene into the *Hprt* locus (achieved by insertional targeting). Staining of mammary glands from this line revealed less than 1% staining, mainly limited to the ducts and a few end buds. Our assessment of this line of mice is that the recombination frequency was too low for this to be used for genetic screens.

(iii) *Hprt-CMV-cre allele*. This allele was generated by inserting CMV-cre into the *Hprt* locus. This was not expected to be mammary specific, however we hoped that this line would express at a lower level than the *ß–casein* Cre allele. However this mouse line exhibits high levels of cre recombination both in somatic tissues and the germ line. This mouse strain was described in *Genesis* 32, 197-188, 2002.

D. Generation of pre-deletion cell lines

In preparation for *in vivo* analysis we have generated multiple ES cell lines with double targeted *loxP* endpoints that can be used for generating deletions in vivo. These cell lines have been tested for their germ line transmission potential:

1. *Wnt 3 – E₂DH,*   2.7 Megabases
2. *Chad – Mpo,*   6.8 Megabases
3. *HoxB9 - E₂DH,*   4.9 Megabases
4. *HoxB9 – Chad,*   1.7 Megabases
5. *Pitpn – Mpo,*   12.3 Megabases
6. *p53 – Pitpn,*   6.2 Megabases

The total distance covered by these deletion endpoints is 34.6 Megabases

(7) KEY RESEARCH ACCOMPLISHMENTS

- Long range tissue specific *cre* recombination achieved at 10% efficiency *in vivo*.
- Large inversions generated in ES cells.
- Five different inversions on two chromosomes established in mice.
- F our germ line mammary cre alleles generated.
- Reporter gene evaluation of cre alleles revealed lack of specificity.
- Multiple germ line transmissible double *loxP* ES cells generated on Chromosome 11 covering 34.6 Megabases (about 1.5% of the mouse genome).

(8) REPORTABLE OUTCOMES

Manuscripts published:

- Zheng B, Sage M, Sheppeard EA, Jurecic V & Bradley A (2000). Engineering mouse chromosomes with *Cre-loxP*: Range, efficiency and somatic applications. *Mol. Cell Biol,* 20, 648 – 655.
- Zheng B, Sage M, Cai W-W, Thompson DM, Tavsanli BC, Cheah Y and Bradley A (1999). *Nature Genetics* 22, 375-378.
- Yu Y and Bradley A (2001). Engineering chromosomal rearrangements in mice. *Nature Reviews* 2, 780-790.
- Zheng B, Mills AA & Bradley A (2001). Introducing defined chromosomal rearrangements into the mouse genome. *Methods* 24, 81-94
- Hong Su, Alea Mills, Xiaozhong Wang & Allan Bradley (2002). A targeted X-linked CMV-cre line. *Genesis* 32, 187-188.

Patents
   -none

Degrees.
- Dr. Binhai Zheng's Ph.D work was partially supported by this grant. Dr. Zheng graduated in1999.

- Dr. Hong Su's Ph.D. work was partially supported by this grant. Dr. Su graduated in 2000.

Employment/research opportunities received

- Dr. Binhai Zheng received a postdoctoral training position and fellowship support to join the laboratory of Marc Tessier-Levine in University of California, SanFrancisco.

- Dr. Hong Su, joined Regeneron Pharmaceuticals Inc (New York) as a scientist.

(9) CONCLUSIONS

It has been established that long range cre-loxP recombination is efficient *in vivo*. However, the desired specificity of the Cre has not yet been obtained. We have recognized that there are various developmental problems associated with heterozygosity of several of the deletions listed above. It is therefore necessary to develop more cre lines to obtain the desired specificity before proceeding with the in vivo deletion studies.

If mammary specific long-range recombination is achieved we have the experimental tools to induce tissue specific segmental losses in a region extending 34 Megabases. This is potentially a very powerful genetic system for isolating mammary specific tumor suppressor genes.

Engineering Mouse Chromosomes with Cre-*loxP*: Range, Efficiency, and Somatic Applications

BINHAI ZHENG,[1] MARIJKE SAGE,[1] ELIZABETH A. SHEPPEARD,[1] VESNA JURECIC,[1] AND ALLAN BRADLEY[1,2]*

Department of Molecular and Human Genetics[1] and Howard Hughes Medical Institute,[2]
Baylor College of Medicine, Houston, Texas 77030

Chromosomal rearrangements are important resources for genetic studies. Recently, a Cre-*loxP*-based method to introduce defined chromosomal rearrangements (deletions, duplications, and inversions) into the mouse genome (chromosome engineering) has been established. To explore the limits of this technology systematically, we have evaluated this strategy on mouse chromosome 11. Although the efficiency of Cre-*loxP*-mediated recombination decreases with increasing genetic distance when the two endpoints are on the same chromosome, the efficiency is not limiting even when the genetic distance is maximized. Rearrangements encompassing up to three quarters of chromosome 11 have been constructed in mouse embryonic stem (ES) cells. While larger deletions may lead to ES cell lethality, smaller deletions can be produced very efficiently both in ES cells and in vivo in a tissue- or cell-type-specific manner. We conclude that any chromosomal rearrangement can be made in ES cells with the Cre-*loxP* strategy provided that it does not affect cell viability. In vivo chromosome engineering can be potentially used to achieve somatic losses of heterozygosity in creating mouse models of human cancers.

Specific chromosomal rearrangements can be engineered in mice to model human chromosomal disorders, such as those associated with deletions or duplications of chromosomal segments (for example, Smith-Magenis syndrome, Downs syndrome, and Charcot-Marie-Tooth type 1A) (5, 7, 10). Chromosomal rearrangements also facilitate genetic studies (2, 14). Inversion chromosomes can be used to establish balanced lethal systems to facilitate stock maintenance. Deletions can be used for mapping and in genetic screens for recessive mutations.

In *Drosophila melanogaster* there is a wealth of chromosomal rearrangements that are widely used as genetic tools. In particular, chromosomal deletions (deficiencies) which collectively cover approximately 60 to 70% of the genome have been indispensable in mapping recessive mutations and in region-specific mutagenesis screens. The use of deletions in mice, however, has been much more limited because of the paucity of chromosomal deletions which, until recently, were restricted to a few regions of the mouse genome flanking visible genetic markers (14). The application of the Cre-*loxP* recombination system over large distances in mouse embryonic stem (ES) cells has made it possible to engineer specific chromosomal rearrangements in the mouse (13, 17). This chromosome engineering strategy involves three manipulation steps in ES cells (see Fig. 1): (i) one *loxP* site is targeted to one endpoint along with the 5′ half of an *Hprt* selectable marker gene (5′ *hprt*); (ii) another *loxP* site is targeted to a second endpoint with the 3′ half of the *Hprt* gene (3′ *hprt*); and (iii) transient expression of Cre recombinase catalyzes *loxP* site-specific recombination, leading to the desired rearrangement. Reconstitution of a full-length *Hprt* gene provides selection for ES cells with the recombination products in culture in HAT (hypoxanthine-aminopterin-thymidine) medium. By using this technology,

deletions, duplications, inversions, or translocations can be generated depending upon the relative position and orientation of the two *loxP* sites and selection cassettes (13, 17).

The Cre-*loxP* chromosome engineering strategy provides a unique and unprecedented opportunity to manipulate the mouse genome. However, several critical questions remain to be answered in order to explore fully the potential of this technology. First, is there any limit as to the kind and size of rearrangements that can be made with this technology? While there are likely to be biological limits in mice, ES cells harboring large chromosomal deletions offer an opportunity to perform haploid genetic screens in vitro. For such applications, the larger the deletion, the more powerful the screen. Second, what is the efficiency of Cre-mediated recombination for substrates of different genetic distances? This will be pertinent to the scope and applicability of this technology. Third, can this strategy be used to engineer chromosomes somatically, that is, in a tissue- or cell-type-specific manner without the strong positive selection schemes that are used in cell culture? Tissue-specific deletions also enable recessive genetics to be employed somatically, for instance, to induce loss of heterozygosity (LOH) to model genetic changes in human cancers or to conduct screens for novel tumor suppressor genes in combination with mutagenesis strategies. Somatically induced deletions may avoid the developmental problems associated with larger germline deletions and consequently a larger chromosomal region can be studied in a single animal.

To address these questions, we applied the Cre-*loxP* chromosome engineering strategy to various parts of mouse chromosome 11 (Chr 11) in ES cells and in vivo. With an improved selection cassette, we obtained an 11% deletion efficiency for a two-centimorgan (2-cM; equivalent to 4 Mb) deletion substrate in murine ES cells. Rearrangements of up to three-quarters of Chr 11 have been made, demonstrating that there appears to be no recombination-based restriction as to what type of rearrangements can be made provided that ES cells tolerate the genetic change. We found that the efficiency of Cre-mediated recombination between two *loxP* sites on the

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* Corresponding author. Mailing address: Department of Molecular and Human Genetics/HHMI, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-6671. Fax: (713) 798-8142. E-mail: abradley@bcm.tmc.edu.

same chromosome (*cis*) decreases with increasing genetic distance. We found that large chromosomal deletions may be deleterious to ES cells and that deletions which were lethal to developing embryos could be engineered somatically at high efficiencies, breaking ground for somatic chromosome engineering.

MATERIALS AND METHODS

Construction of targeting vectors. The *Hsd17b1* targeted cell line has been described elsewhere (13). The targeting vectors for *Wnt3* (modified from a previous version [8]) and *p53* have also been described elsewhere (24). All microsatellite markers were targeted with insertion vectors. The targeting vectors for *D11Mit199* and *D11Mit69* were modified from previous versions (8), replacing the mutant 3′ *hprt* cassette with the wild-type sequence.

The *D11Mit142* and *D11Mit71* loci were targeted with insertion vectors generated from a targeting-ready genomic library that contains the puromycin resistance gene, a *loxP* site, 3′ *hprt* cassette, and an agouti coat color transgene in the vector backbone (23). Clones isolated from this library were restriction mapped, and a gap was created in the region of homology which was used as the probe to detect targeting by Southern analysis. The targeting vectors for *D11Mit142* have been described (23). A clone with a 10.9-kb genomic insert at *D11Mit71* was isolated from the 3′ *hprt* library and mapped with several restriction enzymes. The insert consists of two flanking (2.3 and 3.9 kb) and three internal (0.8, 3.3, and 0.6 kb) *Nco*I fragments. The internal fragments were deleted from the clone to create a gap in the region of homology, resulting in targeting vector pTVD11Mit71F. The insert was then flipped by using rare cutter *Asc*I sites that flank the insert, resulting in targeting vector pTVD11Mit71R which was used to deliver the *loxP* site to the *D11Mit71* locus with the reverse orientation. The 3.3-kb internal *Nco*I fragment was used as the probe in mini-Southern analysis to detect gap repair-dependent targeting events (20, 23). This probe hybridizes to a 6-kb and a (weak) 2.8-kb wild-type *Eco*RI fragment and, in targeted clones, an additional 18.6-kb targeted fragment resulting from the insertion of the vector sequence into the targeted locus.

Generation and analysis of chromosomal rearrangements. ES cell cultures, gene targeting, and germ line transmission were performed as described previously (12). AB2.2 ES cells were used in most experiments except in a few cases where a hybrid ES cell line (between 129S7 and C57BL/6-*Tyr*^*-Brd*), ER3.4, was used (E. Regel and A. Bradley, unpublished data). Electroporation of the Cre expression plasmid pOG231 (11), selection of Cre recombination products with HAT medium, and drug (neomycin and puromycin) resistance tests were performed as described earlier (8, 13) with some modifications. ES cells (80% confluent) were passaged 1 day before electroporation and fed with medium 2 h before electroporation. The cells were then trypsinized and resuspended in phosphate-buffered saline (PBS), and cell counting was performed with a Coulter Counter. The cells were again suspended in PBS to make the final cell density of 1.1×10^7 cells/ml. In a typical transient Cre expression experiment, 25 μg of pOG231 (prepared by CsCl centrifugation, unlinearized) was electroporated into 10^7 ES cells in 0.9 ml of PBS. The electroporation was conducted with a Bio-Rad GenePulser and a Gene Pulser cuvette with a 0.4-cm electrode gap at 230 V and 500 μF. Cells (in PBS) were then mixed with M15 medium and plated on two to three plates at different densities. For the *cis* 2-cM substrates, electroporated cells were subject to serial dilution before plating to enable counting of the HAT-resistant colonies. HAT selection was initiated about 48 h after electroporation, maintained for 8 days and released in hypoxanthine thymidine (HT) for 2 days before the colonies were counted and picked. In all experiments a 10^4 dilution was also plated for each cell line under no selection to count and calculate the number of colonies that survived electroporation. Assessed by this procedure, usually about one-third of the cells undergoing electroporation survived and formed colonies on feeder plates in M15 medium. To control between different experiments, a 2-cM double-targeted cell line was included in each experiment as a control for the Cre recombination efficiency, and this efficiency (~11%) has been consistent throughout all of the experiments.

FISH. Metaphase chromosome spreads from ES cells were prepared as described previously (15). Fluorescence in situ hybridization (FISH) was performed with phage or BAC probes according to a standard protocol (3). The *mPer1* phage clone, BAC 330H2, was labeled with digoxigenin and detected by anti-digoxigenin-rhodamine antibody. BAC 293C22 and BAC 330P14 were labeled with biotin and detected with fluorescein isothiocyanate-avidin. BAC 232M23 was labeled with a mixture of digoxigenin and biotin. The chromosomes were stained with DAPI (4′,6′-diamidino-2-phenylindole). The images were taken as monotonic pictures, and the composites were made with artificial coloration for clarity.

PCR and sequence analysis. Primer P$_a$, 5′-AGG ATG TGA TAC GTG GAA GA (*Hprt* intron, forward), and primer P$_b$, 5′-GCC GTT ATT AGT GGA GAG GC (polymerase II promoter in the neomycin resistance gene, reverse), were used to specifically amplify by PCR a fragment containing exon 3 sequence in the 5′ *hprt* cassette. Primer P$_a$ and primer P$_c$, 5′-CCA GTT TCA CTA CTA ATG ACA CA (*Hprt* exon 9, reverse), were used to specifically amplify exon 3 sequence in the 3′ *hprt* cassette. Primer P$_d$, 5′-GCA TTG TTT TGC CAG TGT C (*Hprt* exon 6, reverse), was used to sequence exon 3 in the PCR products.
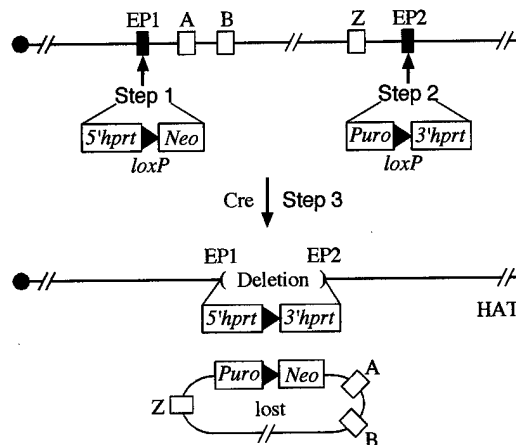

FIG. 1. The Cre-*loxP* based chromosome engineering strategy. 5′ *hprt* was previously named *hprt*Δ3′; 3′ *hprt* was previously name *hprt*Δ5′. A neomycin (*Neo*) or a puromycin (*Puro*) resistance gene is linked to the first or the second *loxP* site, respectively, for positive selection during gene targeting. In this case, Cre recombination between two *loxP* sites targeted in the same orientation in *cis* (on the same chromosome) leads to a deletion that is neomycin and puromycin sensitive due to the loss of the *Neo*- and *Puro*-carrying reciprocal product, a ring chromosome in G_1 (shown) or a duplication sister chromatid in G_2 (not shown). If the two *loxP* sites are on the two different chromosome homologues (in *trans*), a deletion and a duplication will be produced. The rearrangements can then be transmitted through the mouse germline if viable.

The primers used to detect the cardiac specific 2-cM deletion were P1, 5′-CCT CAT GGA CTA ATT ATG GAC (*Hprt* exon 2, forward), and P2, the same as P$_c$ (*Hprt* exon 9, reverse). The primer pair used to detect the αMyHC-Cre (Cre coding sequence under the control of α-myosin heavy chain promoter) transgene has been described elsewhere (1).

RESULTS

High-efficiency Cre-*loxP* based chromosomal engineering with an improved vector in mouse ES cells. Sequence analysis identified a frameshift mutation in the coding portion of the 3′ *hprt* selection cassette (Fig. 2) previously successfully used for chromosome engineering (13), leading to a translation stop codon nine codons downstream of the mutation (Fig. 2A). This mutation should render a reconstituted *Hprt* minigene nonfunctional, yet HAT-resistant colonies were obtained with this cassette. These may have resulted from a repair event during or following Cre recombination (see below). Since the events we have scored to date required selection, the efficiency of Cre-mediated *loxP* site-specific recombination on multimegabase substrates may be greater than that scored by the number of selected HAT-resistant clones. Because the recombination efficiency is pertinent in applications of the Cre-*loxP*-based chromosome engineering strategy, we reassessed this efficiency for a 2-cM interval between *Hsd17b1* (E_2DH) and *D11Mit199* on Chr 11 (8) by using cassettes without the frameshift mutation. The *D11Mit199* locus was retargeted with the corrected 3′ *hprt* cassette in an ES cell line that had been targeted at the *Hsd17b1* locus with the 5′ *hprt* cassette (13) so that the *loxP* sites were in the same orientation (8). The double-targeted cell lines were electroporated with a Cre expression plasmid (pOG231) (11) or a control plasmid (TyBS) (22), and the recombination efficiency was assessed (defined here as the number of HAT-resistant colonies per cell surviving electroporation). No HAT-resistant colony was obtained with the control plasmid. With the Cre expression plasmid, approximately half of the double-targeted clones yielded recombination efficiencies of approximately 11%, while the rest had ef-
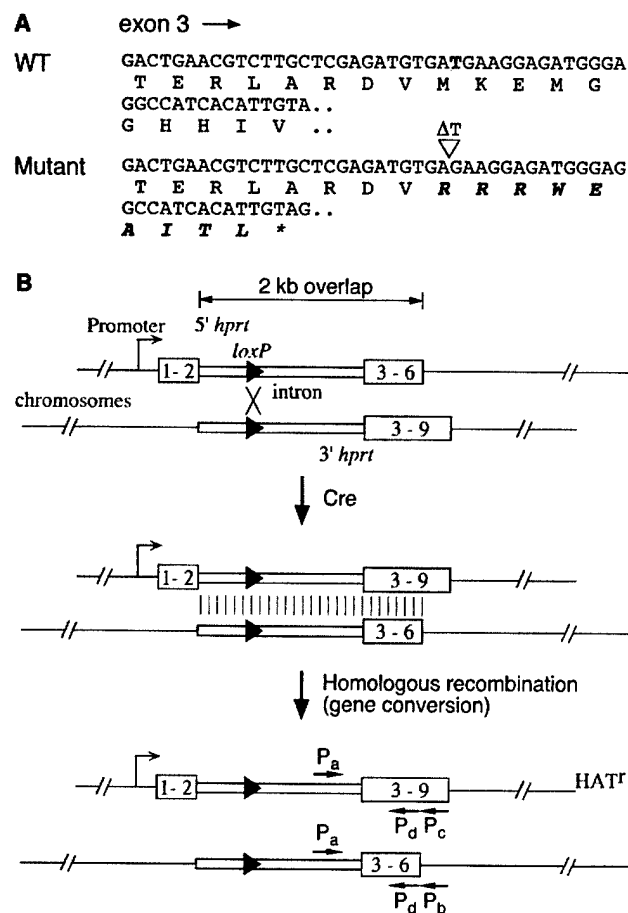
A   exon 3 ⟶

WT   GACTGAACGTCTTGCTCGAGATGTGATGAAGGAGATGGGA
       T   E   R   L   A   R   D   V   M   K   E   M   G
      GGCCATCACATTGTA..
       G   H   H   I   V ..   ΔT
                                                 ▽
Mutant   GACTGAACGTCTTGCTCGAGATGTGAGAAGGAGATGGGAG
          T   E   R   L   A   R   D   V   *R*   *R*   *R*   *W*   *E*
         GCCATCACATTGTAG..
          A   *I*   *T*   *L*   *

B

FIG. 2. A frameshift mutation in the original 3' *hprt* cassette used in chromosome engineering. (A) Partial exon 3 sequence of the wild-type (WT) and mutant 3' *hprt* cassette with conceptual translation. The thymidine residue in the wild-type sequence that is deleted in the mutant is in boldface and underlined. The altered amino acid residues affected by the mutation is in italicized boldface. *, Stop codon. (B) A proposed mechanism by which HAT-resistant colonies were obtained by a combination of Cre-*loxP* site-specific recombination and homologous recombination, as shown for Cre recombination between two *loxP* sites in *trans* in the same orientation that leads to a deletion and a duplication. 1–2, 3–6, and 3–9 refer to *Hprt* exons. P_a, P_b, P_c, and P_d, primers for PCR and sequence analysis of exon 3 of the *Hprt* gene. The portion of the coding sequence affected by the mutation in exons 3–9 is shaded. The polyadenylation signal and neomycin and puromycin resistance genes are not shown for simplicity.

ficiencies of approximately 0.047% (Table 1). Cre recombination products from the former group are puromycin sensitive (indicating that these cell lines have a deletion), while those from the latter group are puromycin resistant (indicating that these cell lines have a deletion and a duplication). Therefore, the two different efficiencies reflect the configuration of the targeted *loxP* sites on the Chr 11 homologues in the parental cell lines, which is resolved into a deletion in *cis* and which is resolved into a deletion and a duplication in *trans* (8, 13). The *cis* events occurred several hundred times more efficiently than the *trans* events. To rule out any effect of HAT selection on Cre-mediated recombination, we transfected three *cis* double-targeted cell lines with the Cre expression plasmid and randomly picked colonies grown under no drug selection. We then determined the percentage of the colonies that were recombined by both drug resistance test and Southern analysis on individual clones. Of 279 colonies picked for all three double-targeted cell lines, 24 had undergone the 2-cM Cre-mediated deletion, yielding a Cre recombination efficiency

of ~9%, which is not significantly different from that assessed by HAT selection. As a control, the 2-cM substrate with the mutant 3' *hprt* cassette gave Cre recombination efficiencies of 0.007% for *cis* and 0.0001% for *trans* (8). Thus, the Cre recombination efficiency is improved by approximately 3 orders of magnitude after correction of the frameshifted 3' *hprt* selection cassette.

Coupled Cre-*loxP* recombination and gene conversion. The *Hprt* cassette reconstructed by Cre-*loxP* recombination from the mutant 3' *hprt* selection cassette should be nonfunctional. However, HAT-resistant colonies were readily obtained (8, 13). This raised the question as to the nature of the event that leads to the HAT-resistant colonies in these experiments. The frequency of spontaneous reversion is too low to explain the observed frequency of HAT-resistant clones from the mutant cassette. The frameshift mutation is located in a 2-kb overlap between the 5' and the 3' *hprt* cassettes, and therefore the mutation in the 3' cassette may be corrected by homologous recombination with sequences in the 5' cassette. We hypothesized that Cre brings the two *loxP* sites together to promote site-specific recombination and that during or immediately after this process the endogenous homologous recombination machinery repairs the mutation (Fig. 2B). This notion would predict that all recombination products would have the wild-type exon 3 sequence rather than a correcting single nucleotide insertion resulting from a spontaneous reversion. Sequence analysis demonstrated that all HAT-resistant colonies had acquired a wild-type sequence in the reconstituted full-length *Hprt* minigene ($n = 10$) (see Fig. 2B and Materials and Methods).

trans recombination events also generate an *Hprt*+ deletion chromosome and the reciprocal product, a duplication chromosome, which retains the recombined overlapping region between the 5' and 3' cassettes (Fig. 2B). Sequence analysis of PCR products from exon 3 in the reciprocal product revealed that this exon 3 remained wild type in all cases analyzed ($n = 17$), indicating that the repair results from a gene conversion event (see Fig. 2B and Materials and Methods).

Long-range chromosomal rearrangements can be made in ES cells. Our chromosome engineering strategy has primarily focused on deletions, duplications, and inversions of a few centimorgans (8, 13). The ability to manipulate a larger region of the chromosome is desirable in many instances. For example, large inversions, when marked with a recessive lethal mutation, can be used as balancer chromosomes (2). ES cells with a large deletion may be useful in screens for recessive mutations in vitro. Since the apparent Cre recombination efficiency was dramatically increased with the corrected 3' *hprt* cassette, we tested whether long-range (defined here as tens of megabases) deletions can be made in ES cells.

A deletion of 22 cM between *Hsd17b1* and *D11Mit69* on Chr 11 was used for this test. Previous attempts to generate this deletion in ES cells with the mutant 3' *hprt* cassette had failed (8). The *D11Mit69* locus was targeted with the 3' *hprt* cassette oriented for a deletion in an ES cell line that had been targeted at *Hsd17b1* (8). Fifteen double-*loxP*-targeted cell lines were transiently transfected with a Cre expression plasmid, and HAT-resistant colonies were counted after 12 days. Drug resistance tests indicated that four parental cell lines were double targeted in *cis* and eleven were double targeted in *trans*. The recombination events were confirmed to be Cre dependent because a mock transfection with a control plasmid (TyBS) yielded no HAT-resistant colonies for one *cis* and one *trans* double-targeted parental cell line. Unlike previous *cis*-*trans* tests, however, Cre recombination for both *cis* and *trans* configurations occurred at a similar efficiency of approximately

TABLE 1. Efficiency of Cre-mediated *loxP* site-specific recombination over different genetic distances

Interval (5' *hprt*-3' *hprt*)	Genetic distance (cM)	Cre recombination efficiency[a] (no. of colonies in HAT/no. of colonies with no drug)		
		loxP sites in opposite orientation: *cis* (inversion)[b] (*n*)	*loxP* sites in the same orientation	
			cis (deletion) (*n*)	*trans* (deletion-duplication) (*n*)
Hsd17b1-D11Mit199	2	ND	$(1.1 \pm 0.5) \times 10^{-1}$ (8)	$(4.7 \pm 0.7) \times 10^{-4}$ (5)
Hsd17b1-D11Mit69	22	ND	$(3.7 \pm 2.4) \times 10^{-5}$ (4)	$(3.1 \pm 1.1) \times 10^{-5}$ (11)
Wnt3-p53	24	$(2.2 \pm 0.6) \times 10^{-3}$ (5)	2.9×10^{-5} (1)[c]	$(8.2 \pm 0.9) \times 10^{-5}$ (5)
Hsd17b1-D11Mit142	30	$(3.2 \pm 0.7) \times 10^{-4}$ (3)	$(9.8 \pm 1.7) \times 10^{-6}$ (2)[c]	$(5.9 \pm 4.7) \times 10^{-5}$ (5)
Hsd17b1-D11Mit71	60	$(8.3 \pm 0.8) \times 10^{-5}$ (2)	$(9.5 \pm 2.1) \times 10^{-7}$ (2)[c]	$(1.4 \pm 0.2) \times 10^{-5}$ (4)

[a] Numbers are the means ± the standard deviations. ND, not determined.
[b] No colonies were obtained with the *trans* configuration because dicentric and acentric chromosomes are produced.
[c] Not confirmed by FISH analysis.

3×10^{-5} (Table 1 and see below). We further successfully generated a number of long-range rearrangements on Chr 11 (Fig. 3; Table 1). The most dramatic example is illustrated in Fig. 4D, where Cre recombination between two *loxP* sites targeted in *trans* to *Hsd17b1* and *D11Mit71* that are 60 cM away from each other on Chr 11 leads to a minideletion chromosome and a large duplication chromosome. Therefore, long-range chromosomal rearrangements, including deletions and deletion-duplications, can be generated with the improved selection cassette.

Large chromosomal deletions may cause ES cell lethality. The Cre-mediated deletion efficiency for the *cis* configuration differs by more than 3 orders of magnitude between a 2-cM (*Hsd17b1-D11Mit199*) and a 22-cM (*Hsd17b1-D11Mit69*) substrate (Table 1). The reduced Cre recombination efficiency for a larger substrate may simply reflect a lower efficiency of Cre-*loxP* juxtaposition with greater physical separation. However, it is also possible that ES cells with larger deletions may be selected against if the deletion has deleterious effects on cell viability or growth. In this scenario, only cells that have undergone a compensatory genetic change would survive. To test this, the deletion cell lines were analyzed by FISH with probes both internal and external to the deletion interval. Intriguingly, of five recombination products derived from three independent *cis* double-targeted parental cell lines, all were trisomy 11, with two wild-type and one deletion chromosome. The two wild-type chromosomes were found to exist as two separate chromosomes (as in Fig. 4B, three of five analyzed) or as a Robertsonian fusion in other cases (as in Fig. 4C, two of five analyzed, both of which derived from independent double-targeted parental cell lines). In contrast, the majority (three of four) of the *trans* recombination products analyzed contain the expected single deletion and duplication chromosomes. The remaining *trans* product contained a duplication chromosome and two deletion chromosomes in the Robertsonian configuration. All double-targeted parental cell lines analyzed, irrespective of the *cis* or *trans* configuration, contain two wild-type chromosomes (data not shown). These results indicate that the deletion in *cis*, which leads to a single copy of the 22-cM region of Chr 11, is haploinsufficient in ES cells. Consequently, rare variants are selected in which the remaining wild-type chromosome is duplicated. Thus, the hemizygous 22-cM deletion causes ES cell lethality or a severe growth disadvantage.

Cre-*loxP* recombination efficiency decreases over increasing genetic distances. The Cre recombination efficiency is an important consideration in designing Cre-*loxP*-based chromosome engineering experiments. To provide a framework for future experiments, we determined this efficiency for *cis* events at different genetic distances. Since a 22-cM deletion had been

observed to cause cell death or a growth disadvantage, we assessed the efficiency of inversions as the indicator of Cre recombination efficiency for the larger intervals. Four rearrangements were included in this analysis: (i) a 2-cM deletion between *Hsd17b1* and *D11Mit199*, Del(11)4Brd; (ii) a 24-cM inversion between *p53* and *Wnt3*, In(11)8Brd; (iii) a 30-cM inversion between *Hsd17b1* and *D11Mit142*, In(11)6Brd; and (iv) a 60-cM inversion between *Hsd17b1* and *D11Mit71*, In(11)7Brd (Fig. 3). When the two *loxP* sites are in opposite orientations, approximately half of the independent double-targeted cell lines give HAT-resistant colonies (interpreted as *loxP* sites in *cis*), and the other half do not give any colonies (interpreted *loxP* sites in *trans*), presumably due to the formation of dicentric and acentric chromosomes. FISH analysis confirmed that the relevant inversions had occurred in representative clones from all three large genetic intervals (data not shown). As shown in Fig. 5, between 2 and 60 cM, the logarithm of the Cre recombination efficiency is inversely proportional to the genetic distance between the *loxP* sites.

Tissue-specific chromosome engineering. Several deletions of a few centimorgans around the *Hsd17b1* locus on Chr 11 are heterozygous lethal (8). Although this underscores the developmental importance of this chromosomal region, lethal deletions cannot be used for genetic screens. However, if the de-
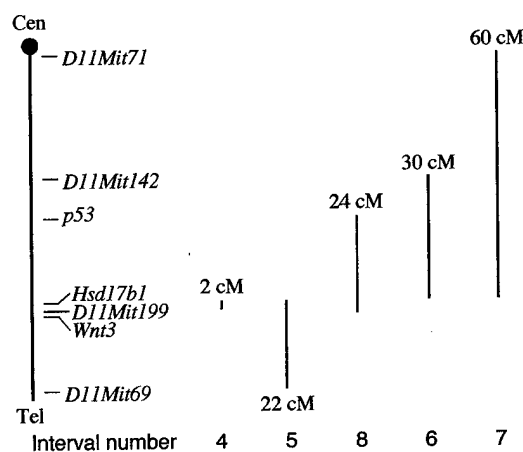

FIG. 3. Genetic intervals of rearrangements made on mouse Chr 11 in this study. 2 cM, *Hsd17b1-D11Mit199*, deletion, deletion-duplication; 22 cM, *Hsd17b1-D11Mit69*, deletion, deletion-duplication; 24 cM, *Wnt3-p53*, inversion, deletion-duplication; 30 cM, *Hsd17b1-D11Mit142*, inversion, deletion-duplication; 60 cM, *Hsd17b1-D11Mit71*, inversion, deletion-duplication. The total genetic distance from centromere (Cen) to telomere (Tel) is about 80 cM.
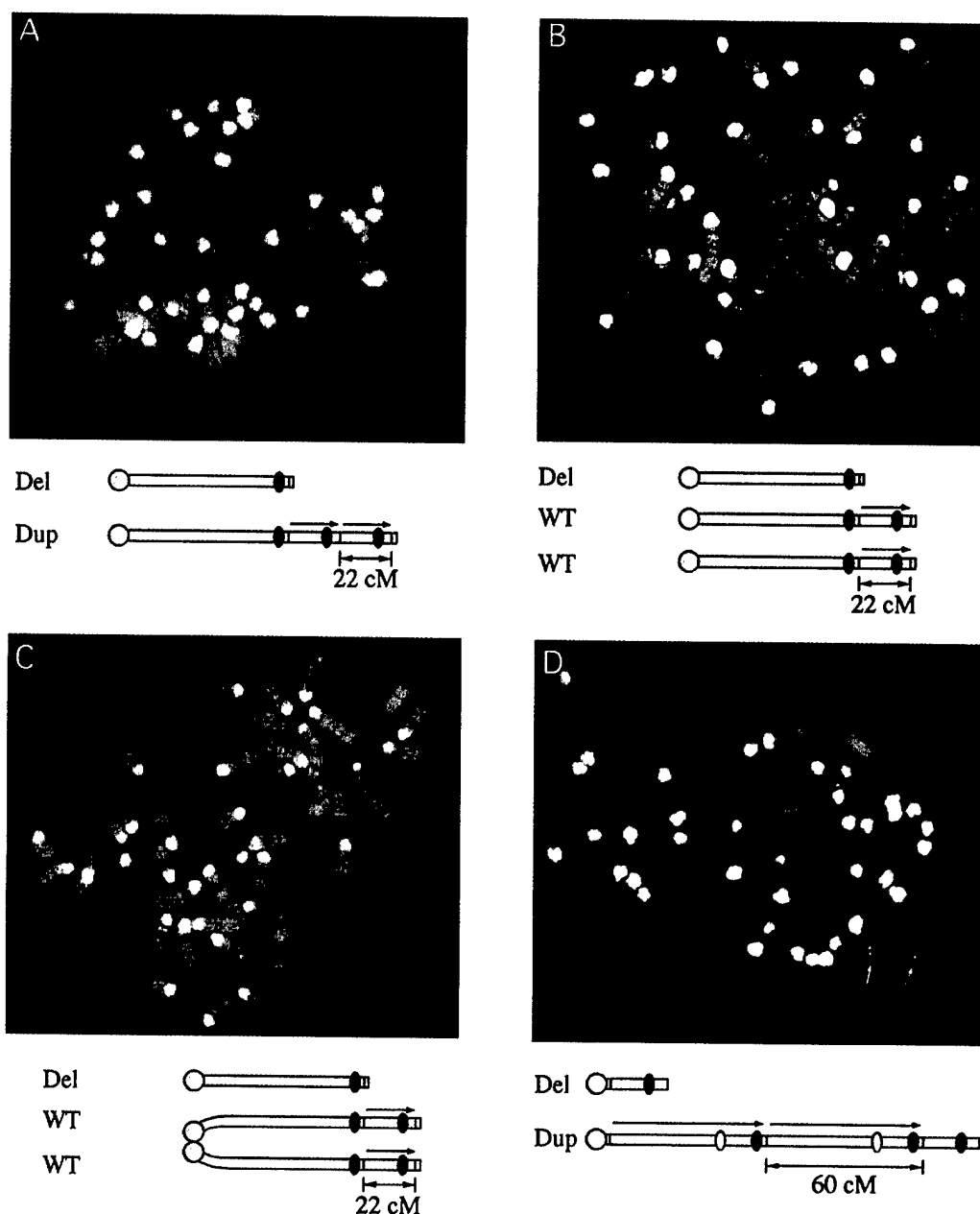
FIG. 4. FISH analysis of long-range Cre recombination products on Chr 11. (A) Del(11)5Brd-Dp(11)5Brd, a 22-cM deletion chromosome and a 22-cM duplication chromosome produced by a *trans* event between *Hsd17b1* and *D11Mit69*. (B) Del(11)5Brd-WT-WT, a 22-cM deletion chromosome produced by a *cis* event between *Hsd17b1* and *D11Mit69*, while the remaining wild-type chromosome is duplicated to survive. (C) The same as in panel B except that the duplicated wild-type chromosomes are in a Robertsonian (or iso-chromosome) configuration. (D) Del(11)7Brd-Dp(11)7Brd, a 60-cM deletion chromosome and a 60-cM duplication chromosome produced by a *trans* event between *Hsd17b1* and *D11Mit71*. Colors: yellow, BAC 232M23 (*D11Mit320*); red, BAC 330H2 (*D11Mit263*); green, BAC 330P14 (*D11Mit11*). Two or more probes were differentially labeled and artificially colored.

letion can be made somatically, for instance, in a tissue- or cell-type-specific manner, the problem of heterozygous lethality can be partially circumvented. To test this possibility, we generated a 2-cM *Hsd17b1-D11Mit199* double-targeted mouse line (deletion substrate) and crossed it to a cardiac-specific Cre (i.e., αMyHC-Cre) (1). The αMyHC-Cre line had previously been used to make cardiac-specific deletions of several kilobases with an efficiency of up to 90% (1). Tissue DNA was isolated from two progeny that inherited both the αMyHC-Cre transgene and the 2-cM substrate. PCR analysis with primers specific to the reconstituted *Hprt* minigene was performed to

determine whether the Cre-mediated recombination had occurred (Fig. 6A). This analysis demonstrated that the Cre recombination occurred in heart, but not in skeletal muscle, liver, lung, or spleen (Fig. 6A). To provide a more quantitative measure of Cre recombination, Southern analysis was performed on two animals by using restriction digestions and a probe at *Hsd17b1* that would distinguish the wild-type allele, the (double) targeted allele and the Cre-recombined allele (Fig. 6B). Deletion occurred exclusively in the heart but not in the other organs tested (Fig. 6B). Based on the ratio of intensity of the recombined fragment and the predeletion allele for
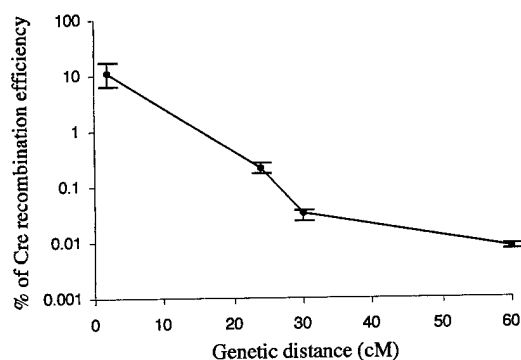
FIG. 5. Efficiency of Cre recombination over genetic distance. The percentage of Cre recombination efficiency (y axis, in $\log_{10}$ scale) is plotted against the genetic distance (x axis, in linear scale). The first datum point represents a deletion. The other three points with larger genetic distances represent inversions. Error bars indicate the standard deviations. The numbers of independent experiments were as indicated in Table 1.

both animals tested, the deletion efficiency in the heart is about 10%.

DISCUSSION

The organism that the Cre-*loxP* system is derived from, bacteriophage P1, evolved the system to resolve its ~100-kb genome into monomeric circular forms (18). The Cre-*loxP* site-specific recombination system has been extensively used for conditional genetic technology, namely, the temporal and spatial control of gene expression in mice (16). In these applications, the genetic material involved (as determined by the distance between the two *loxP* sites) is usually a few kilobases. We have previously shown that this system can be adapted for substrates of several megabases by incorporating a positive selection scheme (8, 13). In the present study, we redefined the Cre recombination efficiency for a 4-Mb substrate, after we corrected a mutation in the selection cassette. Surprisingly, the efficiency for this substrate is approximately 11% by transient Cre expression. This efficiency approaches that obtained with substrates of several kilobases and indicates that at between several kilobases and several megabases the Cre-*loxP* recombination occurs at comparable efficiencies. This might reflect aspects of chromatin domain organization such that sequences that are 1-kb to 1-Mb apart may have similar separations in three-dimensional space. In this aspect, the fact that the 2-cM cardiac-specific deletion can be detected by Southern analysis is of particular significance. In many cancers, interstitial deletions are the dominant mode for loss of the remaining allele of a tumor suppressor gene (6). Therefore, in vivo chromosomal deletions can be used to mimic somatic LOH in human cancers and in searches for novel tumor suppressor genes in combination with point mutagenesis.

The 22-cM deletion between *Hsd17b1* and *D11Mit69* on Chr 11 appears to cause ES cell lethality or a severe growth disadvantage because deletion products for this interval exclusively carry an additional wild-type Chr 11. This may be due to a dosage effect of one or multiple genes in this interval such that a single copy of these genes cannot support the normal growth of ES cells (haploinsufficiency). The Cre-*loxP*-mediated deletion of this 22-cM region therefore selects for cells that have duplicated the wild-type Chr 11. This result underscores the tight control of the euploid ES cell genome. A region of haploinsufficiency has also been proposed to reside on Chr 9 in studies on a radiation-induced deletion complex (19). The ob-
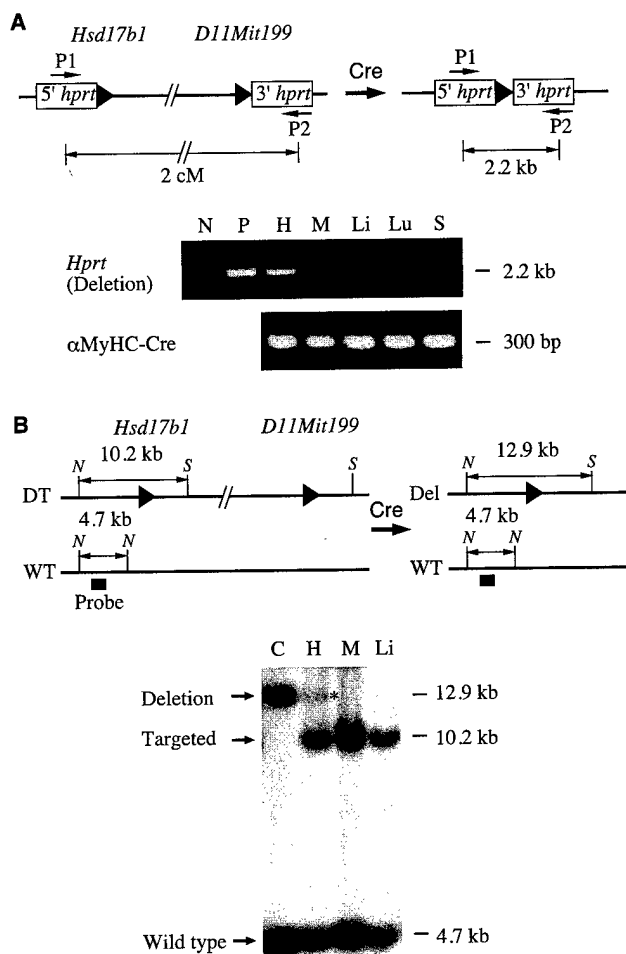

FIG. 6. Cardiac-specific 2-cM deletion between *Hsd17b1* and *D11Mit199*. (A) PCR analysis of the deletion products. P1 and P2, primers used to specifically amplify the full-length *Hprt*. PCR reactions on αMyHC-Cre serve as a control. N, negative control; P, positive control; H, heart; M, skeletal muscle; Li, liver; Lu, lung; S, spleen. (B) Southern analysis of the deletion products. C, control cell line that contains a deletion and a wild-type chromosome. The cardiac-specific deletion band is indicated by an asterisk. *N*, *Nhe*I; *S*, *Sfi*I; solid triangle, *loxP* site.

servation of haploinsufficiency in ES cells is in direct contrast with many cancer cells that often carry large chromosomal deletions and chromosomal losses. Such a unique feature of ES cells may be further studied by isolating suppressors of this lethality caused by the deletions. On the other hand, these data indicate that duplications are tolerated better than deletions in ES cells. This is consistent with the notion that monosomies rarely, if ever, exist, whereas trisomy 8, 11, and 15 and several other chromosomes have been observed in ES cells (9). The relatively frequent occurrence of trisomy 11 is further suggested by our observation that one of four 22-cM deletion-duplication products analyzed by FISH contain one duplication and two deletion chromosomes where the deletion chromosome is presumably not required to be duplicated for cell survival or growth. The lethality caused by large deletions in ES cells precludes a straightforward approach of using the deletion as a partial haploid reagent in mutagenesis screens.

It is possible that the partial-trisomy ES cells selected by the 22-cM *cis* deletion are derived from an underlying trisomy 11 population in the ES cells transfected with Cre. Although these cells are not detected by analysis of double-targeted clones,

extrapolation of the inversion recombination efficiencies suggests that either these cells are present at 10^{-2} frequencies in the transfected clones or that this nondisjunction event is induced by the Cre-*loxP* recombination event itself.

Large-deletion-associated ES cell lethality can obscure the Cre recombination efficiency. We therefore determined the Cre efficiency by using large inversion substrates. This analysis indicates that Cre recombination efficiency decreases over increasing genetic distances. However, in all cases, the recombination products (HAT-resistant colonies) are readily obtained in sufficient numbers in a single experiment except when inviable products are generated (dicentric and acentric chromosomes). For multimegabase substrates, the logarithm of the Cre recombination efficiency is approximately inversely proportional to the genetic distance (Fig. 5). This can be used as a guide for future experiments with Cre-*loxP*-based chromosome engineering. However, other factors, such as chromosomal locations and differences in experimental manipulations, may affect the Cre recombination efficiency. For deletions, the Cre recombination drops more precipitously as the genetic distance increases for two reasons. First, the physical barrier Cre has to overcome to bring the two *loxP* sites together is greater as the distance between the two *loxP* sites increases, as in inversions. Second, larger deletions may cause ES cell lethality or a growth disadvantage and are consequently selected against after Cre recombination. In the *trans* configuration where a deletion and a duplication chromosomes are the products, Cre recombination efficiency is moderately reduced with an increasing genetic distance (Table 1). This suggests that chromosome homologues may pair in a mitotic cell cycle, assisting Cre recombination by bringing the two *loxP* substrates on different chromosomes to the same subcellular location. Under such circumstances, the closer the two *loxP* sites are genetically, the closer they are physically when the two chromosomes pair, and therefore, the higher the Cre recombination efficiency. Alternatively, if *trans* recombination occurs mainly in G_2 and recombined sister chromatids tend to segregate away from each other, as reported in *Drosophila* (4), the HAT-resistant deletion products will frequently contain a wild-type chromosome instead of the duplication chromosome. In this scenario, *trans* deletion-duplication events involving a larger distance will appear to occur at a lower frequency due to the production of haploinsufficient deletions.

The Cre recombination efficiency for large deletion-duplications is probably comparable to that for translocations between nonhomologues. In some of our experiments, we analyzed some random integration clones when targeting the second *loxP* site. Upon Cre expression, approximately half of these clones give HAT-resistant colonies and the other half do not give viable HAT-resistant colonies. The former group presumably yields translocations, while the latter group yields dicentric and acentric products. The efficiency of generating these translocations is about 10^{-5}. It has been reported using a similar strategy that Cre recombination efficiency for a translocation between chromosomes 12 and 15 occurs at about 10^{-7} (17). The higher efficiency in our experiments may be due to the Cre plasmid, the tissue culture conditions, and/or the electroporation procedures used. It remains possible that the 2-kb homology between our 5' *hprt* and 3' *hprt* cassettes assists the Cre-*loxP* recombination by recruiting the homologous recombination machinery to help secure the *loxP* site recombination synapse.

The mutant 3' *hprt* cassette used in previous chromosome engineering experiments provides a unique opportunity for studying a potential interaction between homologous and site-specific recombination. Sequence analysis of Cre recombina-

tion products indicates that the mutation is repaired by homologous recombination with the wild-type template in the 5' *hprt* cassette. This homologous recombination cannot occur in the absence of site-specific recombination since the homology is only 2 kb, but the substrates are on different chromosomes or far away (multimegabases) from each other on the same chromosome. Therefore, it must have occurred during or immediately after the Cre-*loxP* recombination. It is possible that the Holliday junction structure created by Cre (21) can be resolved by homologous recombination machinery. This scenario would suggest that the two recombination events are not mutually exclusive and can be coupled under specific circumstances. The other possibility is that Cre-*loxP* recombination facilitates gene conversion merely by bringing the two substrates together. Immediately after site-specific recombination, homologous recombination occurs. Since HAT-resistant colonies for a 2-cM substrate are obtained with an efficiency of approximately 3 orders of magnitude higher with the wild-type 3' selection cassette than with the mutant version, homologous recombination responsible for repairing the mutation occurs ca. 0.1% of the time after Cre recombination.

Taken together, the Cre-*loxP* chromosome engineering strategy provides a powerful tool for genetic studies and for genome manipulation. We explored the possibility and determined the efficiency of generating various chromosomal rearrangements on mouse Chr 11. We conclude that any desired rearrangement can be made with the Cre-*loxP* system provided that the rearrangement does not have any deleterious effect on the ES cells. Cre-*loxP* recombination is very efficient for substrates of a few centimorgans both in tissue culture and in vivo. This efficiency decreases over increasing genetic distances between the two *loxP* sites. The work presented here provides a framework for future applications of chromosome engineering.

ACKNOWLEDGMENTS

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Engineering a mouse balancer chromosome

Binhai Zheng[1], Marijke Sage[1], Wei-Wen Cai[1], Debrah M. Thompson[1,2], Beril C. Tavsanli[3], Yin-Chai Cheah[1] & Allan Bradley[1,2,3]

Balancer chromosomes are genetic reagents that are used in *Drosophila melanogaster* for stock maintenance and mutagenesis screens[1]. Despite their utility, balancer chromosomes are rarely used in mice because they are difficult to generate using conventional methods. Here we describe the engineering of a mouse balancer chromosome with the Cre-*loxP* recombination system. The chromosome features a 24-centiMorgan (cM) inversion between *Trp53* (also known as *p53*) and *Wnt3* on mouse chromosome 11 that is recessive lethal and dominantly marked with a *K14-Agouti* transgene[2]. When allelic to a wild-type chromosome, the inversion suppresses crossing over in the inversion interval, accompanied by elevated recombination in the flanking regions. The inversion functions as a balancer chromosome because it can be used to maintain a lethal mutation in the inversion interval as a self-sustaining *trans*-heterozygous stock. This strategy can be used to generate similar genetic reagents throughout the mouse genome. Engineering of visibly marked inversions and deficiencies is an important step toward functional analyses of the mouse genome and will facilitate large-scale mutagenesis programs.

Balancer chromosomes enable the maintenance of lethal mutations without selection in *Drosophila*[3]. The rationale is as follows: for a lethal mutation in gene *A*, *lA*, an *lA*/+ × *lA*/+ cross will yield progeny in the ratio of 1 *lA/lA* (inviable):2 *lA*/+:1 +/+. Because *lA*/+ and +/+ progeny are often indistinguishable, maintaining *lA* requires constant selection or genotyping. By introducing another lethal mutation, *lB*, to the homologous chromosome, *lA* is maintained in a *trans*-heterozygous state, *lA* +/+ *lB*. An *lA* +/+ *lB* × *lA* +/+ *lB* cross will always produce *lA* +/+ *lB* progeny so long as there is no recombination between the two loci (*lA* +/*lA* + and + *lB*/+ *lB* progeny will also be produced, but are inviable). To prevent recombination from occurring, a crossover suppressor must be present. This is most conveniently achieved by an inversion (or a complex of inversions) on the chromosome carrying *lB* (the balancer), because a single crossover between an inversion and a normal chromosome gives inviable dicentric and acentric products or aneuploidy[4] (Fig. 1). A self-perpetuating *lA* +/+ *lB* stock constitutes a balanced lethal system. In addition to *lB*, the inversion is typically marked with a dominant marker so that progeny carrying the balancer are readily identified. Because balancer chromosomes suppress recombination, they are used to maintain the integrity of mutagenized chromosomes and are therefore crucial reagents for mutagenesis screens[1]. For instance, in intercrosses between siblings that have inherited the same balancer chromosome and the mutagenized chromosome, absence of non-balancer–carrying progeny (as assessed by the dominant marker) indicates the presence of one or more recessive lethal mutations on the mutagenized chromosome.

In the mouse, advances in N-ethyl-N-nitrosourea (ENU) mutagenesis[5–7] and positional cloning methods[8,9] have presented large-scale mutagenesis as a viable approach for functional genomics. Such phenotype-driven mutagenesis screens make no assumption about the gene products to be analysed and therefore will identify novel pathways, novel genes and novel functions of known genes. A model study at a region surrounding the mouse albino locus has yielded a wealth of functional genomic information for this region[10–12]. Most regions of the mouse genome are not accessible for such analyses, however, due to a lack of marked chromosomal rearrangements. In this study, we tested the idea of engineering a marked inversion as a balancer chromosome in the mouse.

We made a 24-cM inversion between *Trp53* and *Wnt3* on mouse chromosome 11. The conserved linkage to a gene-rich segment on human chromosome 17 (ref. 13) makes this region a suitable target for large-scale mutagenesis efforts. We chose *Wnt3* as an endpoint because a *Wnt3* mutation confers homozygous lethality[14]. We chose the genetic distance to be sufficiently large to be useful for a considerable region of chromosome 11 (24 cM/80 cM≅30%), and sufficiently small to minimize double crossovers that obviate the balancing effect. We used the Cre-*loxP* chromosome engineering strategy[15] to generate the inversion in mouse embryonic stem (ES) cells by successive gene targeting of a *loxP* site to the two endpoints, followed by Cre-mediated recombination between the two *loxP* sites leading to the desired
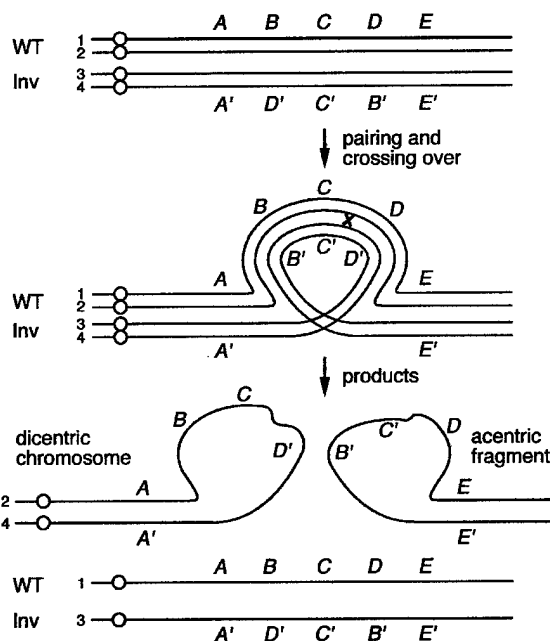

Fig. 1 How a single crossover between a wild-type chromosome and a paracentric inversion (an inversion that does not span the centromere) leads to inviable dicentric and acentric products. WT, wild type; Inv, inversion. The four chromatids are numbered. 'x' indicates the site of a crossover. Adapted from ref. 4.

[1]Department of Molecular and Human Genetics, [2]Howard Hughes Medical Institute and [3]Developmental Biology Program, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. Correspondence should be addressed to A.B. (e-mail: abradley@bcm.tmc.edu).
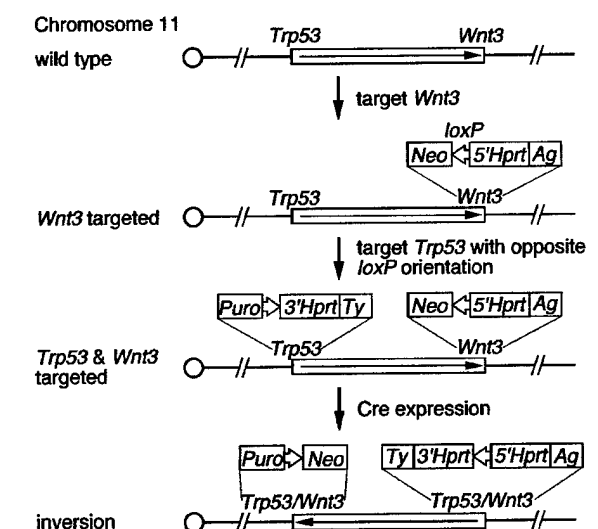
letter

Fig. 2 A three-step Cre-*loxP*-based strategy to engineer the *Trp53-Wnt3* inversion. *Neo*, neomycin resistance gene; *Puro*, puromycin resistance gene; *Ag*, *K14-Agouti* transgene; *Ty*, tyrosinase minigene; 5′ *Hprt* or 3′ *Hprt*, 5′ or 3′ half of an *Hprt* minigene. For simplicity the other chromosome 11 homologue (wild type) is not shown.

rearrangement (Fig. 2). Reconstitution of a full-length *Hprt* gene from the two non-functional halves that are linked to either *loxP* site provides a selection for the recombination products in HAT medium[15]. By incorporating coat-colour markers into the target-

ing vectors, the inversion is visibly marked. We targeted *Wnt3* with a dominant *K14-Agouti* coat-colour gene that has been shown to confer a yellowing of the coat colour on an otherwise wild-type agouti mouse[2,16]. We targeted *Trp53* with a tyrosinase (*Tyr*) minigene that has been shown to give pigment to an otherwise albino mouse[17].

We targeted both *Wnt3* and *Trp53* with replacement vectors designed to inactivate the genes (Fig. 3). The targeted *Wnt3* allele, *Wnt3^Brdm2*, was constructed to orient the 5′ *Hprt-loxP* cassette toward the centromere[18] (Fig. 3*d*). As the transcript orientation of *Trp53* relative to the centromere was unknown, we constructed two targeting vectors for *Trp53* with opposite *loxP*-site orientations (Fig. 3*a*, and data not shown). The targeted *Trp53* alleles, *Trp53^Brdm2* and *Trp53^Brdm3*, were obtained in heterozygous *Wnt3^Brdm2* ES cell lines. We electroporated six *Trp53^Brdm2_ Wnt3^Brdm2* double-targeted cell lines with a Cre expression plasmid and obtained HAT-resistant colonies from five of these cell lines (6.0±0.4×10³ (mean±s.e.m.) HAT-resistant colonies per 10⁷ electroporated cells). This suggested that the HAT-resistant colonies were inversions resulting from recombination between two *loxP* sites on the same chromosome in opposite orientations, whereas the remaining parental cell line carried the two *loxP* sites on different chromosome homologues leading to inviable dicentric and acentric chromosomal fragments upon Cre recombination[15]. Drug-resistance tests indicated that the HAT-resistant colonies were resistant to both neomycin and puromycin, as expected for inversion products. Southern-blot analysis using probes at either junction region detected the expected restriction fragments (Fig. 3), indicating that a precise rearrangement had occurred. Fluorescence *in situ* hybridizations (FISH) on metaphase chromosomal spreads confirmed that an inversion
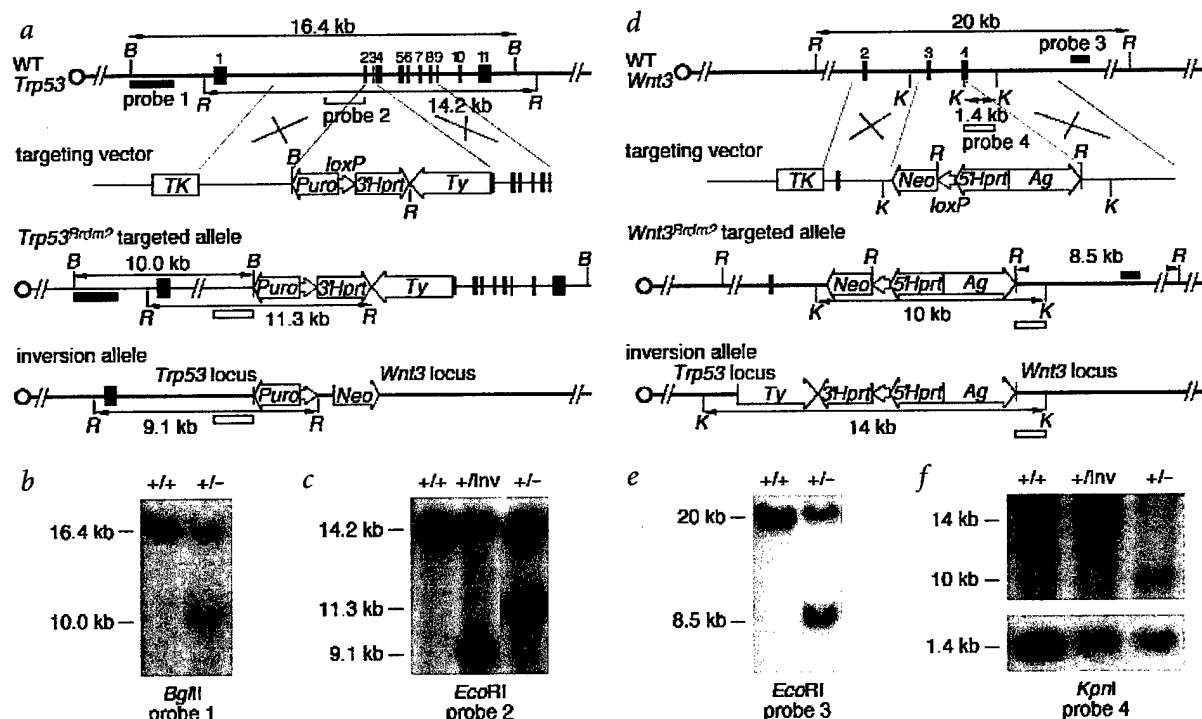

Fig. 3 Southern-blot analysis of the *Trp53^Brdm2* targeted allele, the *Wnt3^Brdm2* targeted allele and the *Trp53-Wnt3* inversion. *a*, Genomic structure of wild-type *Trp53* locus, targeting vector, predicted *Trp53^Brdm2* targeted allele and inversion allele. Exons are indicated by vertical black bars. *TK*, herpes simplex virus thymidine kinase gene; *Neo*, neomycin resistance gene; *Puro*, puromycin resistance gene; *Ty*, tyrosinase minigene; *Ag*, *K14-Agouti* transgene. *B*, *Bgl*II; R, *Eco*RI, K, *Kpn*I. *b*, Southern-blot analysis to detect the *Trp53^Brdm2* allele using probe 1. *c*, Southern analysis to distinguish between wild-type, targeted and inversion alleles using probe 2. *d*, Genomic structure of wild-type *Wnt3* locus, targeting vector, predicted *Wnt3^Brdm2* targeted allele and inversion allele. *e*, Southern-blot analysis to detect the *Wnt3^Brdm2* allele using probe 3. *f*, Southern analysis to distinguish between wild-type, targeted and inversion alleles using probe 4.
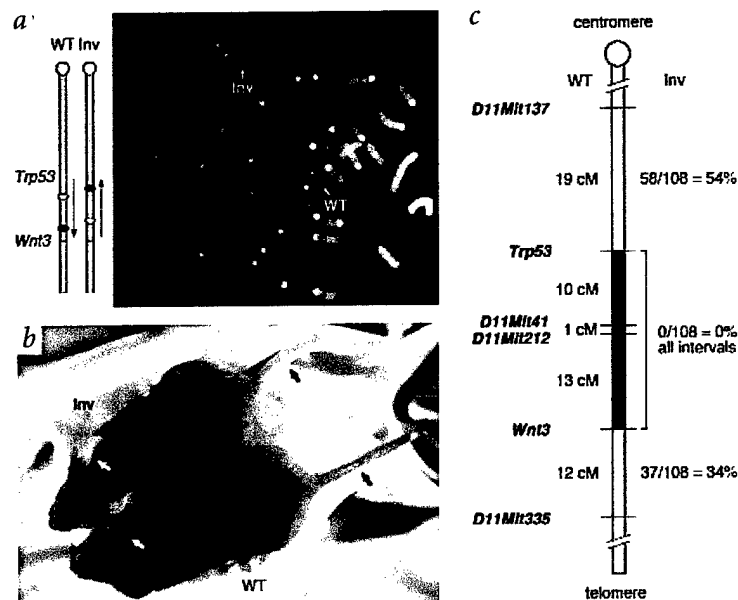
Fig. 4 Characterization of the *Trp53-Wnt3* inversion. **a,** FISH analysis of the *Trp53-Wnt3* inversion. WT, wild type; Inv, inversion. Yellow, a *D11Mit320* BAC probe; red, a *D11Mit263* BAC probe. The parental double-targeted cell lines showed a wild-type order of the two probes on both chromosome 11 homologues (data not shown). **b,** Expression of the dominant marker *K14-Agouti* in the *Trp53-Wnt3* inversion. The back of the earlobes and the tails are indicated with open and filled arrows, respectively. **c,** Recombination frequencies in and around the *Trp53-Wnt3* inversion. The established genetic distances in wild type are marked in cM (left). The observed recombination frequencies with the inversion are shown (right). The inversion is indicated by a filled bar.

was obtained (Fig. 4*a*). In contrast, the *Trp53^{Brdm3}-Wnt3^{Brdm2}* double-targeted cell lines gave rise to duplication/deletion products, as expected for recombination between two *loxP* sites in the same orientation (data not shown).

We next transmitted ES cells harbouring the *Trp53-Wnt3* inversion, In(11*Trp53*; 11*Wnt3*)8Brd, through the mouse germ line via standard procedures[19]. Chimaeric males were mated with C57BL/6-*Tyr^{c-Brd}* (albino) females. Among the (agouti) germline pups, approximately one-half were wild-type agouti and the other half had lighter tail and ear colouring (Fig. 4*b*), which we confirmed to be due to the expression of the *K14-Agouti* transgene. Except for the coat-colour difference, inversion progeny were phenotypically indistinguishable from wild-type littermates. Preliminary studies on homozygous inversion embryos indicate they have the same phenotype as *Wnt3*-null mutants[14].

To test the expression and transmission of the coat-colour markers, we backcrossed (129S7×C57BL/6-*Tyr^{c-Brd}*) F₁ heterozygous inversion males (heterozygous for the albino and agouti loci) to three different inbred strains: 129S5 (agouti), C57BL/6J (black) and C57BL/6-*Tyr^{c-Brd}* (albino). In all cases, a lighter tail colour co-segregated with the inversion (as determined by Southern-blot analysis) in an agouti or black background (Table 1). Thus, *K14-Agouti* functions as a dominant marker in a non-albino background. Among the albino progeny from the C57BL/6-*Tyr^{c-Brd}*×F₁ matings, there was no detectable difference in coat colour between the inversion mice and wild-type littermates, indicating that the *Tyr* minigene on the inversion chromosome was not expressed.

To examine whether the inversion suppresses crossing over in the inversion interval, we determined the recombination frequencies on chromosome 11 by scoring the backcross progeny. In addition to Southern-blot analysis with *Trp53* and *Wnt3* probes at the inversion junctions (Fig. 3), we analysed four polymorphic SSLP markers that lie proximal (*D11Mit137*), internal (*D11Mit41, D11Mit212*) and distal (*D11Mit335*) to the inversion (Fig. 4*c*). The two internal markers map close to the centre of the inversion and were used to screen for double crossovers. Among all 108 meioses examined, there was no recombination within the inversion, indicating successful suppression of recombination (Fig. 4*c*). Because of chiasma interference, the frequency of double crossovers is presumably lower than 1%. In contrast, the flanking region showed elevated recombination frequencies (Fig. 4*c*), possibly as a mechanism to compensate for the loss of recombination in the inversion interval[20].

To test whether the inversion indeed functions as a balancer chromosome, we crossed the inversion to a recessive lethal mutation, *Hoxb4^r* (ref. 21), which lies in the inversion interval. We intercrossed mice *trans*-heterozygous for the inversion and the *Hoxb4^r* mutation, collected the progeny and genotyped them at weaning age. Of four litters from two matings, all were heterozygous for both the inversion and the *Hoxb4^r* mutation (Fig. 5). The average litter size was four, as expected if one-half the pups were inviable.

Our results demonstrate that large inversions compatible with normal development can be engineered in ES cells and established in the mouse. The *Trp53-Wnt3* inversion can be used to maintain any recessive lethal, sterile or other detrimental mutations in the 24-cM interval, such as mutations in *Brca1* or the *Hoxb* series. It can be used in chromosome 11 region-specific mutagenesis screens[22]. To create a balancer for the entire chromosome, multiple inversions must be generated with a combination of strategies such as mutant *loxP* sites[23,24] or a different site-specific recombination system[25,26]. The strategy described here can also be used to engineer marked deficien-

Table 1 • Coat-colour segregation of inversion backcrosses

Parental genotypes (female×male)	Agouti progeny		Black progeny		Albino progeny	
	WT normal tail	Inv light tail	WT normal tail	Inv light tail	WT	Inv
129S5×F₁(Inv)	18	21	–	–	–	–
C57BL/6J×F₁(Inv)	12	4	9	5	–	–
C57BL/6-*Tyr^{c-Brd}*×F₁(Inv)	9	3	4	3	11	9

The genotypes at the albino locus (or *Tyr; C*) and the agouti locus (*A*) are: *CCAA* for 129S5; *CCaa* for C57BL/6J; *ccaa* for C57BL/6-*Tyr^{c-Brd}*. 129S7 and 129S5 are identical, except at the *Hprt* locus. F₁ refers to (129S7×C57BL/6-*Tyr^{c-Brd}*) F₁.
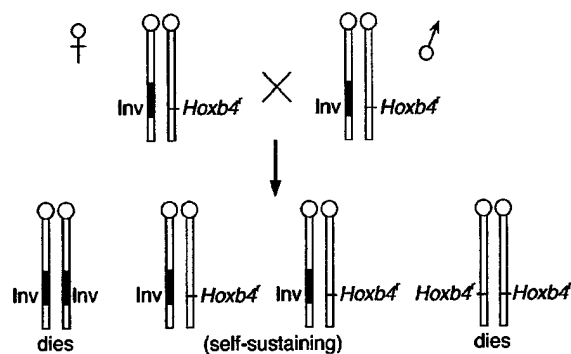
Fig. 5 Use of the *Trp53-Wnt3* inversion to balance the *Hoxb4[r]* lethal mutation.

cies. The ability to engineer marked deficiencies and balancer chromosomes will greatly facilitate genetic studies in the mouse.

Methods

Generation of the *Trp53-Wnt3* inversion mice. We constructed the *Wnt3* targeting vector by modifying a previous vector[18] such that correct targeting would replace a 2.1-kb fragment containing exon 3 and most of exon 4 with a cassette containing a neomycin resistance gene, a *loxP* site, 5′ *Hprt* (also known as *hprtΔ3′*; ref. 15) and the *K14-Agouti* transgene[2]. We generated chimaeric mice from two *Wnt3[Brdm2]* targeted ES cell lines, both of which gave germline transmission. The two cell lines behaved the same in later experiments and are therefore not distinguished in the text. To obtain isogenic genomic DNA to construct *Trp53* targeting vectors, we isolated a clone from an arrayed 129S5 genomic phage library using primer pairs amplifying a fragment of *Trp53* that contains an intron to distinguish between *Trp53* and its pseudogene[27]. A phage clone was obtained, mapped and found to contain exons 1–9. Another *NcoI* site lies 4 kb upstream of the *NcoI* site in exon 2 that is absent in a published map[28], possibly due to a polymorphism. We constructed a replacement vector with a selection cassette containing a puromycin-resistance gene, a *loxP* site, 3′ *Hprt* (after correcting a mutation in the coding sequence of the original cassette, B.Z. and A.B., manuscript

submitted; also known as *hprtΔ5′*; ref. 15) and the *Tyr* minigene[17] using the 4-kb *NcoI* fragment as the 5′ homology arm and a 2.6-kb *SacI* fragment as the 3′ homology arm (Fig. 3a). Correct targeting was expected to delete a 0.6-kb genomic fragment between the *NcoI* site in exon 2 and the *SacI* site in exon 4. We constructed a second replacement vector with the entire selection cassette in the reverse orientation (data not shown).

We performed ES cell cultures, electroporation, mini-Southern-blot analysis on ES cell colonies and generation of chimaeric and germline mice as described[19,29]. We used AB2.2 ES cells that were derived from a 129S7 male embryo. For Cre expression, we electroporated 10[7] ES cells with plasmid pOG231 (25 µg) and initiated HAT selection after 48 h (ref. 15). Drug-resistance tests on Cre recombination products were performed as described[15,18]. We introduced the *Hoxb4[r]* mutation from a 129S5 inbred background.

Fluorescence *in situ* hybridization. We prepared metaphase chromosome spreads from ES cells as described[30]. FISH was performed following a standard protocol[31]. Two BAC clones that map within the inversion were differentially labelled and used as the probe.

PCR polymorphic analysis. We used four SSLP markers[8] polymorphic between 129S5 and C57BL/6 (H. Su and A.B., unpublished data). The products were analysed on a 4% agarose gel (3:1 high resolution blend, Amresco). The sizes of the amplified products are (in the order of 129S5 or 129S7, C57BL/6): *D11Mit137*, 152 bp, 138 bp; *D11Mit41*, 158 bp, 136 bp; *D11Mit212*, 164 bp, 148 bp; *D11Mit335*, 134 bp, 120 bp. The genetic distances on the wild type chromosome were derived from chromosomal positions (in cMs) in the Chromosome Committee Reports (http://www.informatics.jax.org/bin/ccr/index).

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http://www.hgmp.mrc.ac.uk/Biology/resources_index.html
RecA web site:
http://www.tigr.org/~jeisen/RecA/RecA.html
Research Genetics BAC and PAC resources:
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ENGINEERING CHROMOSOMAL REARRANGEMENTS IN MICE

Yuejin Yu* and Allan Bradley‡

The combination of gene-targeting techniques in mouse embryonic stem cells and the Cre/*loxP* site-specific recombination system has resulted in the emergence of chromosomal-engineering technology in mice. This advance has opened up new opportunities for modelling human diseases that are associated with chromosomal rearrangements. It has also led to the generation of visibly marked deletions and balancer chromosomes in mice, which provide essential reagents for maximizing the efficiency of large-scale mutagenesis efforts and which will accelerate the functional annotation of mammalian genomes, including the human genome.

MOUSE GENOMIC TECHNOLOGIES ◎

SEGMENTAL HAPLOIDY
When a diploid organism is haploid for a certain chromosomal region after its deletion or loss.

BALANCER CHROMOSOME
A chromosome with one or more inverted segments that suppress recombination. They are used as genetic tools because they allow lethal mutations to be maintained without selection.

**Program in
Developmental Biology,
Department of Molecular
and Human Genetics,
Baylor College of Medicine,
One Baylor Plaza, Houston,
Texas 77030, USA.
‡The Sanger Centre,
Hinxton, Cambridge
CB10 1SA, UK.
e-mails: yyu@bcm.tmc.edu;
abradley@sanger.ac.uk*

The mouse has become an important model for studying genetics and disease because it shares physiological, anatomical and genomic similarities with humans. In both organisms, alterations of chromosomal structure can occur spontaneously or after exposure to specific DNA-damaging agents, causing, in many cases, significant biological consequences. In humans, chromosomal abnormalities are a principal cause of fetal loss and developmental disorders[1,2], and chromosomal translocations are involved in the genesis of many types of human tumour[3]. Chromosomal rearrangements in mice can be used to model these diseases and enable the fine genetic dissection of their causes.

Chromosomal deletions[4,5], duplications[6], inversions[7] and translocations[8] can be induced in mice by using radiation or chemical mutagens, such as chlorambucil[5]. Some useful rearrangements have been induced using these approaches, one of which has served as a mouse model of trisomy 21 (REFS 6,9). Deletions that overlap a handful of mouse chromosomal loci, such as the albino and pink-eyed dilution loci on chromosome 7, have been used for fine genetic mapping and genetic screens[10,11]. However, the usefulness of radiation or chemical mutagens for inducing rearrangements is limited by the fact that the end points of the induced rearrangements cannot be predetermined.

Strategies have recently been developed to introduce defined chromosomal rearrangements into the mouse genome by engineering them in embryonic stem (ES) cells using the Cre/*loxP* site-specific recombination system[12] (BOX 1). Using these strategies, mouse models that accurately recapitulate human chromosomal rearrangements have been developed[13-18]. These engineered mouse models, together with the availability of the human genome sequence, will significantly enhance our ability to uncover the specific molecular mechanisms of the defects caused by human chromosomal rearrangements.
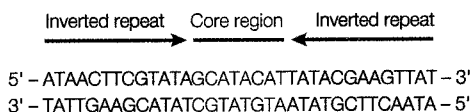
Chromosomal engineering technology has also led to the generation of novel genetic reagents for the functional analysis of the mouse genome. Deletion chromosomes that are visibly marked by, for example, coat-colour markers, can be engineered to provide SEGMENTAL HAPLOIDY in the diploid mouse genome. Recessive mutations that are induced in these deletion intervals from mutagenesis experiments can be detected by crossing mutant mice to mice that are hemizygous for different regions of the genome[12,19]. Mouse BALANCER CHROMOSOMES have also been developed using Cre/*loxP* technology by tagging chromosomal inversions with recessive lethal mutations and coat-colour markers[20]. As inversions suppress recombination, these balancer chromosomes can be used to

Box 1 | Cre/*loxP* site-specific recombination

The reaction catalysed by the P1 bacteriophage Cre recombinase leads to site-specific recombination between two *loxP* sites[51,52].

Inverted repeat   Core region   Inverted repeat

5' – ATAACTTCGTATAGCATACATTATACGAAGTTAT – 3'
3' – TATTGAAGCATATCGTATGTAATATGCTTCAATA – 5'

The *loxP* sequence consists of two 13-bp inverted repeats and an 8-bp asymmetrical core spacer region, which determines the orientation of the site (as shown). The recombination reaction is initiated by Cre binding specifically to the inverted repeat sequences at *loxP* sites, which leads to the formation of a synapse that consists of four Cre subunits and two *loxP* sites in the same orientation. Cre catalyses exchange between the pair of sites in the core spacer region by concerted cleavage and rejoining reactions. A *cis* recombination event between two *loxP* sites in the same orientation will lead to the excision of the *loxP*-flanked DNA sequence as a circular molecule. If *loxP* sites are orientated in opposite directions, the *loxP*-flanking sequence will be inverted. Recombination between two *loxP* sites *in trans* will lead to the reciprocal exchange of the regions that flank the *loxP* sites. Cre can also induce these recombination events when the *loxP* sites are located several megabases apart on the same chromosome, or on two homologous or non-homologous chromosomes[12,24,38,39].

CROSSING OVER
The exchange of genetic material between two homologous chromosomes.

BLASTOCYST
A preimplantation embryo that contains a fluid-filled cavity called a blastocoel.

POSITIVE SELECTION
When a specific chemical is added to a culture medium, the cells that express a positive selectable marker gene, such as the neomycin or puromycin resistance genes, survive and are selected for.

HPRT MINIGENE
(Hypoxanthine phosphoribosyl transferase gene). This is divided into two complementary, but non-functional, fragments: 5'*Hprt* contains exons 1–2 and 3'*Hprt* contains the remaining exons, 3–9. Each *Hprt* fragment is linked to a *loxP* site, and Cre-mediated recombination unites the 5' and 3' cassettes, and restores Hprt activity, which is required for purine biosynthesis and allows desired recombination events to be selected for in HAT (hypoxanthine, aminopterin and thymidine) medium.

prevent CROSSING OVER in an inverted region — a property that can be used to facilitate large-scale mutagenesis screens[21] (as discussed in more detail below). In this review, we discuss the experimental strategies associated with these recent advances and the contributions that mice with engineered chromosomes are making to functional genomics, and to the study of human genetics and diseases such as cancer.

Chromosomal engineering

The strategy of chromosome engineering is based on the techniques of gene targeting in ES cells and the Cre/*loxP* system (FIG. 1). Using gene targeting, two *loxP* sites are inserted sequentially into two loci in the ES-cell genome. The transient expression of the gene that encodes Cre recombinase in double-targeted ES cells induces recombination between the two targeted *loxP* sites to generate the rearranged chromosome. Using various methods, such as drug selection, Southern blot analysis and fluorescent *in situ* hybridization (FISH), the ES-cell clones that carry the desired chromosomal rearrangement are identified and characterized. Chimaeras are generated by injecting these ES cells into mouse BLASTOCYSTS, from which the progeny that carry engineered chromosome are derived (FIG. 1).

Deletions, duplications and inversions. Defined chromosomal deletions, duplications and inversions are important rearrangements not only because they constitute prevalent classes of genomic anomaly in humans, but also because they provide powerful reagents for mouse functional genomics. Generating these types of genomic alteration begins with defining the two end points of the rearrangement. For regions greater than 1 Mb, end points might be selected from more than 6,000 simple sequence length polymorphism (SSLP) markers that have been mapped in the mouse genome (see link to STS Physical Map of the Mouse at the Whitehead Institute). The primers that

are designed to amplify these markers can be used to isolate genomic clones for constructing the end-point targeting vectors[22]. Genes might also be used as end points using high-resolution mapping information that is available for the mouse genome[23] (see link to Genetic and physical maps of the mouse genome at the Whitehead Institute). Both SSLP markers and genes have successfully been used as the end points for engineering numerous chromosomal rearrangements[12,24,25].

In the first step of Cre/*loxP*-mediated chromosomal engineering, a *loxP* site, a POSITIVE SELECTION cassette, one of two complementary but non-functional fragments of a hypoxanthine phosphoribosyl transferase (*Hprt*) gene[12] are introduced into the first end point, in the ES-cell genome, by gene targeting (FIG. 2). To accomplish this, gene-targeting vectors, such as those shown in FIG. 2 are required. These targeting vectors can either be generated in the conventional way, by sequentially inserting various genetic components into a plasmid construct[12,26], or they can be isolated directly from genomic libraries of pre-made targeting vectors[19]. The targeting vectors from these libraries contain all the genetic elements that are required for chromosome engineering, as illustrated in FIG. 2, they require a minimal amount of manipulation before use and they are available from A.B.

ES-cell clones with a *loxP* site targeted to a first end point can be identified by positive selection and by Southern blot analysis[27]. The subsequent procedures used in our laboratory for generating deletions, duplications and inversions are outlined in FIGS 3 and 4. Only ES-cell lines with an inactivated *Hprt* gene, such as the AB2.2 line[28], can be used in these procedures (see link to the Cell Line Request Form for more information on accessing these ES-cell lines). This is because the Cre/*loxP*-mediated recombination event generates a functional *HPRT* MINIGENE, which is used to select ES-cell clones that contain the desired rearrangement (see below). After isolating the clones targeted at a first end point, a second *loxP* site and the complementary *Hprt* fragment are targeted to a second end point. About six to eight double-targeted clones need to be identified by using the positive selectable markers in the second targeting vector and by Southern blot analysis. We expect half of these clones to be targeted on the same chromosome (*in cis*) as the original targeted insertion, whereas the other half will be targeted to the homologous chromosome (*in trans*).

The type of chromosome rearrangement derived from double-targeted cells will be determined by the *loxP* configuration (see supplementary Table 1 online for the possible outcomes of Cre-mediated recombination), which depends on the orientation of the *loxP* site in a targeting vector. To induce *loxP* recombination, a *cre*-expression vector, such as pOG231 (REF. 29), is electroporated into double-targeted clones. Recombination between the *loxP* sites unites the 5'*Hprt* and 3'*Hprt* cassettes and reconstitutes a functional *Hprt* gene. Culturing these ES cells in a medium that contains hypoxanthine, aminopterin and thymidine (HAT) selects for clones that carry the functional *Hprt* gene, and therefore the rearranged chromosomes.

If the relative orientations of the two end-point loci (with respect to the centromere) are known, a specific *loxP* configuration can be designed. If the orientations of the two loci are unknown, as is the case for many chromosome-engineering projects, targeting vectors with different orientations of *loxP* sites will need to be tested (FIGS 3 and 4). The complexity of the recombination products that are generated when the orientations
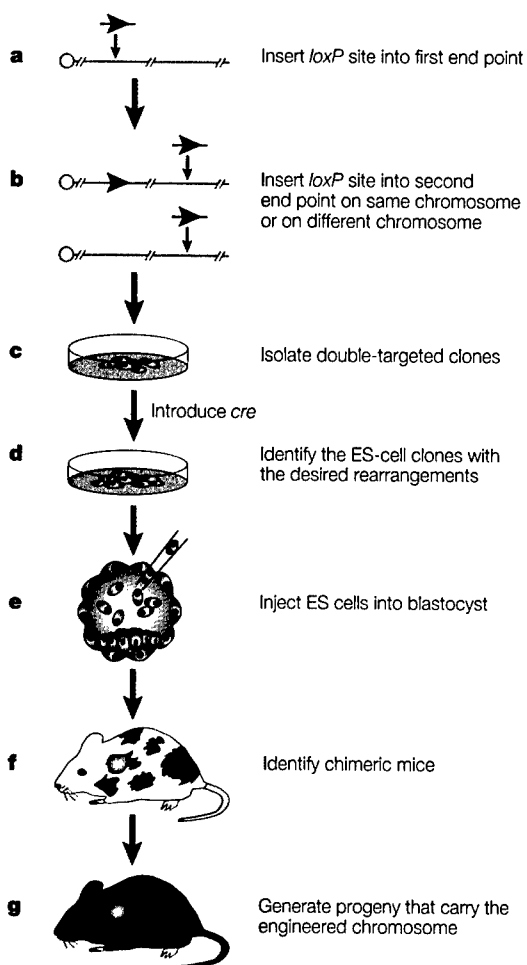

Figure 1 | **A general strategy for chromosomal engineering in mice. a** | A *loxP* site is inserted into the first end point in the embryonic stem (ES)-cell genome using a targeting vector that carries a positive selectable marker gene. **b** | A second *loxP* site, linked to a different positive selectable marker gene, is targeted to the second end point, either on the same chromosome or on a different chromosome by gene targeting or by random insertion. **c** | The expression of *cre* in double-targeted ES cells catalyses recombination between *loxP* sites at the rearrangement end points. **d** | ES-cell clones that carry the desired chromosomal rearrangements are identified and molecularly characterized. **e** | The selected ES cells are injected into mouse blastocysts and the embryos are transferred into the uteri of pseudopregnant foster mothers. **f** | Chimaeras that are generated from blastocyst injection are mated with wild-type mice to establish germ-line transmission of the modified genome. **g** | The progeny derived from the chimaeras are characterized, and a mutant mouse line that carries an engineered chromosome is established.
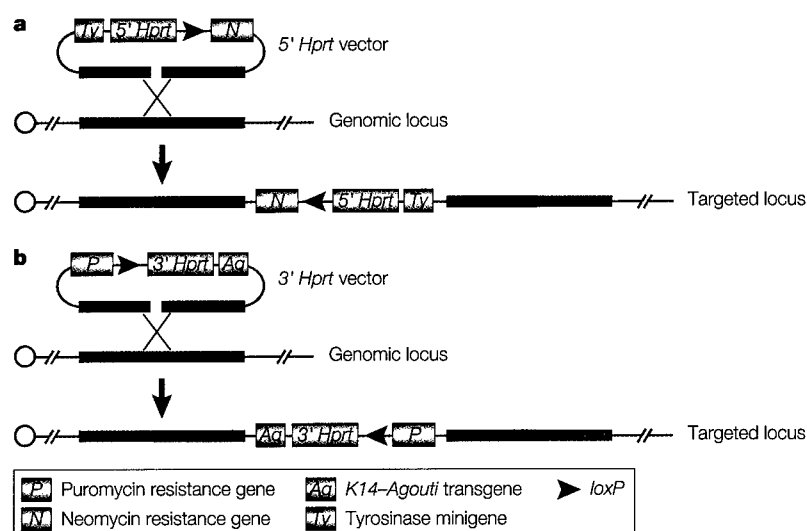
and orders of the selection cassettes are unknown is considerable (see FIGS 3 and 4, and supplementary Table 1 online).

To obtain clones that carry a chromosomal deletion and/or a chromosomal duplication, the two targeted *loxP* sites should be orientated in the same direction. If the *loxP* sites are orientated in opposite directions, Cre-mediated recombination between *loxP* sites *in cis* and *in trans* will generate an inversion and inviable recombination products (ACENTRIC and DICENTRIC chromosomes), respectively (FIG. 4 and supplementary Table 1 online). Therefore, if after *cre* expression, HAT-resistant colonies are not recovered from some of the double-targeted clones, this usually indicates that the two targeted *loxP* sites are located in opposite orientations. Given such an observation, deletions and duplications can be generated by inverting the *loxP* selection cassette and re-targeting the second end-point vector.

Cre-mediated recombination can occur in a cell at the G1 phase of the cell cycle or after DNA replication has occurred (S/G2). After chromosome replication, four *loxP* sites will be present in the double-targeted ES-cell genome, and Cre-mediated recombination can occur between sister or non-sister chromatids depending on whether the *loxP* sites are inserted *in cis* or *in trans*. These post-replication events might lead to several recombination outcomes (as shown in FIGS 3 and 4, and supplementary Table 1 online), some of which will not survive selection in HAT medium.

We have found that, when the *cre* expression vector — pOG231 — is used, the efficiency of Cre-mediated *cis* recombination is ~10% and does not alter appreciably if the distance between two targeted *loxP* sites is changed from a few kilobases to up to 10 Mb (REF. 25) (Y.Y. and A.B., unpublished data). When the *loxP* sites are on homologous chromosomes (*trans*), recombination is approximately two to three orders of magnitude less efficient[25] than when they are *in cis*. So, when *loxP* sites are believed (or known) to be in the same orientation, it is possible to identify double-targeted clones in which the *loxP* sites are inserted *in cis* or *in trans* by analysing recombination efficiencies. Selection analysis of the HAT-resistant clones with G418 and puromycin can also be used to classify the clones that carry various types of chromosomal rearrangement. These rearrangements can then be further analysed by Southern blot analysis and by FISH using mouse bacterial artificial chromosome (BAC) clones as probes.

The same strategy can be used to generate inversions (FIG. 4), although in this case the two *loxP* sites remain at the end points of the rearrangement after an inversion has been generated. In principle, the inverted region could revert back to its non-inverted state; however, although this might occur in a small percentage of cells, these cells will not survive in the HAT selection medium.

Variations of the aforementioned strategy have been reported by other groups[24,30–32]. Besides pOG231, several other *cre*-expression vectors, such as pBS185

Figure 2 | **Gene targeting in embryonic stem cells.** Insertional targeting vectors, as shown, can be used to insert *loxP* sites, positive selectable markers, the *Hprt* gene fragments and coat-colour markers (such as *Ty* and *Ag*) to predetermined loci in the embryonic-stem-cell genome. **a** | Expression of the neomycin resistance and **b** | puromycin resistance genes allows different targeting events to be selected. The complementary, but non-functional, 5′*Hprt* and 3′*Hprt* fragments are derived from a *Hprt* minigene[12]. The dark blue and light blue bars represent regions of homology between the vector and the genomic locus. The vector is linearized in the region of homology (gap) to stimulate targeted insertion into the locus. X represents recombination between the vector and the genome. *Ag*, the *K14–AGOUTI* transgene; *Hprt*, hypoxanthine phosphoribosyl transferase; *Ty*, the TYROSINASE minigene.

TYROSINASE
Tyrosinase is required for melanin biosynthesis, and the expression of its gene leads to pigment production, and is therefore used as a coat-colour marker.

HSVTK
The herpes simplex virus thymidine kinase (HSVtk) is essential for thymidine nucleotide biosynthesis through a salvage pathway and is often used as a negative selectable marker in gene targeting.

NEGATIVE SELECTABLE MARKER
A negative selectable marker gene, such as *HSVtk*, allows cells that express it to be killed when a specific chemical is added to a culture medium, whereas cells that no longer express the marker gene survive.

HAPLOINSUFFICIENCY
A phenotype that arises in diploid organisms owing to the loss of one functional copy of a gene.

HYBRID ES-CELL LINE
An embryonic stem (ES) cell line isolated from F₁ hybrid embryos, such as from crosses between the strains C57BL/6-Tyrᶜ¹ᴮʳᵈ × 129S7 or 129S1 × CAST/Ei. These lines facilitate simple sequence length polymorphism analysis.

(REF. 24), pBS500 (REF. 30) and pIC-*CRE*[32], have been used in chromosomal-engineering experiments in ES cells. Other strategies also select for the desired recombination products in different ways. In some cases, a herpes simplex virus thymidine kinase (HSVTK) gene, which acts as a NEGATIVE SELECTABLE MARKER, is inserted between the rearrangement end points. Cre/*loxP*-mediated recombination events that result in a deletion and the loss of this marker can be selected by culturing ES cells in medium that contains 1,2′ deoxy-2′-fluoro-β-D-arabinofuranosyl-5-iodo-uracil[24,30–32], which kills cells that express HSVtk. However, negative selection cannot be used to isolate duplications or inversions. Positive-selection strategies facilitate the isolation of cells with reciprocal recombination products (such cells would not survive negative selection), which allows ES cells with balanced genetic changes to be recovered. These rearrangements can then be assessed independently of each other after their segregation in the germ line of mice. This is particularly useful because duplications can rescue mice that inherit a corresponding HAPLOINSUFFICIENT deletion.

One factor that limits the generation of deletions in ES cells is the size of the rearranged interval. Available evidence indicates that large deletions, such as those deletions larger than 22 cM, might lead to ES-cell lethality or to a severe growth disadvantage of the cells in culture[25]. Although Cre/*loxP* recombination will occur readily over these large distances, clones will often emerge from these experiments that have undergone a compensatory genetic change, such as a chromosomal duplication[25].

Nested chromosomal deletions. These are a series of overlapping deletions that surround a predetermined genomic locus. These deletions vary in size and have different end points (nested end points), but many of them will overlap. If the genomic locations of the end points are known, nested deletions can be extremely useful for mapping novel recessive mutations. By crossing mice that carry a hemizygous deletion with a mouse line that carries a novel recessive mutation, progeny that harbour both the deletion and the novel mutation *in trans* can be generated. If a recessive mutant phenotype is observed in the progeny of such a cross, it indicates that the chromosome that carries the deletion cannot complement the novel mutation; the novel mutation is therefore located in the deletion interval. Using this approach, novel mutations can be rapidly mapped to a specific deletion interval by crossing mutant mice with mice that carry nested deletions.

To efficiently engineer these types of reagent, we have developed an approach for constructing deletion complexes that does not require that targeting vectors be made for the nested end points[33]. Deletion complexes can be anchored to a predetermined location in the genome by targeting the 5′*Hprt–loxP* cassette as described previously. The 3′*Hprt–loxP* cassette is then inserted randomly into the ES-cell genome by retrovirus-mediated integration (FIG. 5), which generates a library of ES clones with the same targeted end point and a collection of random end points. Only a subset of random insertions will occur on the same chromosome as the original targeting event. However, Cre/*loxP* recombination efficiency is several orders of magnitude more efficient when *loxP* sites are inserted on the same chromosome. So, after the expression of *cre*, most HAT-resistant clones will be derived from retroviral insertions that have occurred *in cis* to the targeted insertion. Cre/*loxP* recombination efficiencies will also decrease if the sites are separated by more than 10 Mb. So, most HAT-resistant clones will have rearrangements that are less than 10 Mb. Clones that are generated by using this strategy carry a random distribution of deletion sizes that range from a few kilobases to several megabases[33]. Clones that contain chromosomal deletions lose the *neo* and *puro* cassettes, and so can be distinguished from other types of rearrangement by sib-selection in G418 and puromycin.

This nested deletion strategy has also been repeated using electroporation to insert the *loxP* cassette randomly into the ES-cell genome[34]. Compared with retrovirus-mediated integration, insertion by electroporation might increase the risk of genomic rearrangements occuring at the insertion site and tandem repeats of a vector might be introduced into the insertion site, although these should be reduced to a single locus by the activity of Cre on a head-to-tail concatenate.

Deletions that are generated by the random insertion of the second end point are usually characterized by Southern blot analysis and by FISH and, if a HYBRID ES-CELL LINE is used, by SSLP analysis. The end points can be defined by cloning the genomic DNA that flanks the deletion end points and by mapping these junction fragments onto a physical map of the region.

Nested deletions have also been generated by irradiation[35-37]. Deletions that are induced by irradiation can be localized and made selectable by targeting a vector that carries a negative selection cassette (such as *HSVtk*) to a predetermined locus. Before irradiation, the cells can be cultured under positive selection pressure to retain the targeted locus. After irradiation, clones that carry the desired deletion can be identified by loss of the negative selection marker. Using this approach, deletions can be produced efficiently; however, they require extensive additional characterization to define each deletion interval.

Chromosomal translocations. One of the main reasons for engineering defined chromosomal translocations is to develop mouse models for human translocations, which underlie certain forms of cancer by causing the abnormal expression of cellular oncogenes or by creating novel fusion genes[3].
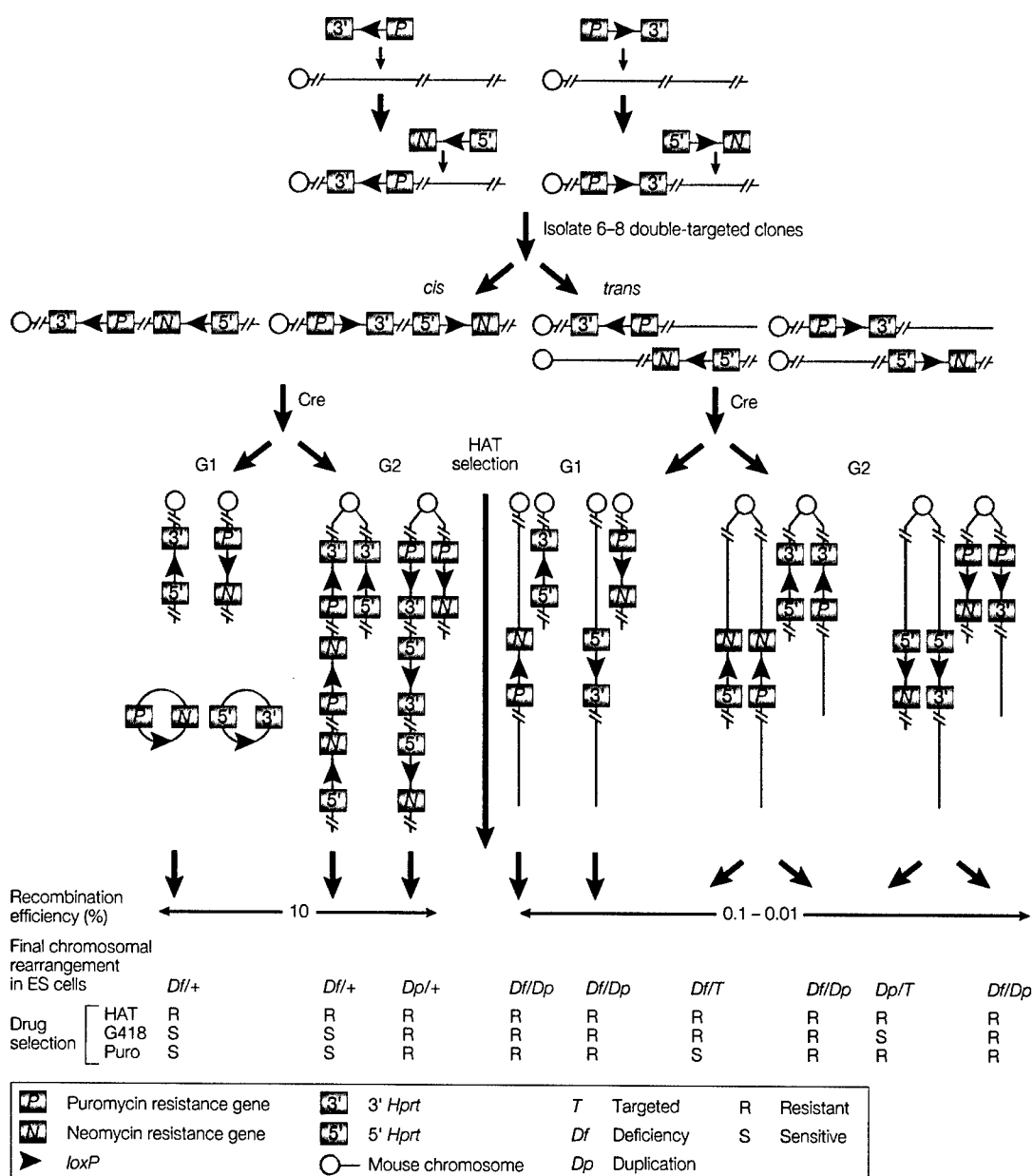

Figure 3 | **Engineering a deletion and/or a duplication in embryonic stem cells.** An experimental procedure for engineering chromosomal deletions and duplications. The cassettes can be targeted in two orientations, only orientations that result in deletions or duplications are illustrated. G1 and G2 indicate the different phases of the cell cycle in which recombination occurs. In G2, four *loxP* sites are located on duplicated chromatids, and recombination events will result in various products. Drug selection will help with identifying the desired rearrangements. For clarity, only the drug-resistance characteristics of the HAT-resistant clones are shown. After *trans* recombination in G2, the chromosome that carries the *Hprt* resistance marker will either segregate with the reciprocal product (carrying the duplication) to give a *Df/Dp* cell, or it will segregate with a non-recombined chromatid that carries the targeting vector (*T*). These cells (*Df/T* or *Dp/T*) are therefore resistant to either G418 or puromycin (Puro) but not both. HAT, hypoxanthine, aminopterin and thymidine; *Hprt*, hypoxanthine phosphoribosyl transferase.

Mouse chromosomal translocations with predetermined breakpoints have been created using Cre/loxP recombination[15,16,38,39]. Translocations are generated when loxP sites are targeted to non-homologous chromosomes. To obtain the desired chromosomal translocation, these targeted loxP sites need to be orientated in the same direction relative to their respective centromeres. If two targeted loxP sites are in opposite orientations, recombination will result in acentric and dicentric chromosomes (FIG. 6a). The efficiency of Cre/loxP recombination between non-homologous chromosomes is several orders of magnitude lower than that of the recombination between loxP sites on the same chromosome. The frequency of Cre/loxP-mediated recombination between non-homologous

chromosomes is also lower than that obtained when loxP sites are inserted within a few megabases of each other on homologous chromosomes.

To generate a fusion protein from a chromosomal translocation, the targeting vectors need to be specially designed so that after translocation, two genes originally located on two non-homologous chromosomes can be linked through their introns, with the loxP site embedded in the junction region of the breakpoint. After RNA splicing, an in-frame fusion mRNA and protein are generated as a result (FIG. 6b). To prevent the generation of acentric and dicentric chromosomes, only pairs of genes with the same transcriptional orientations relative to their centromeres can be engineered to generate fusion proteins.
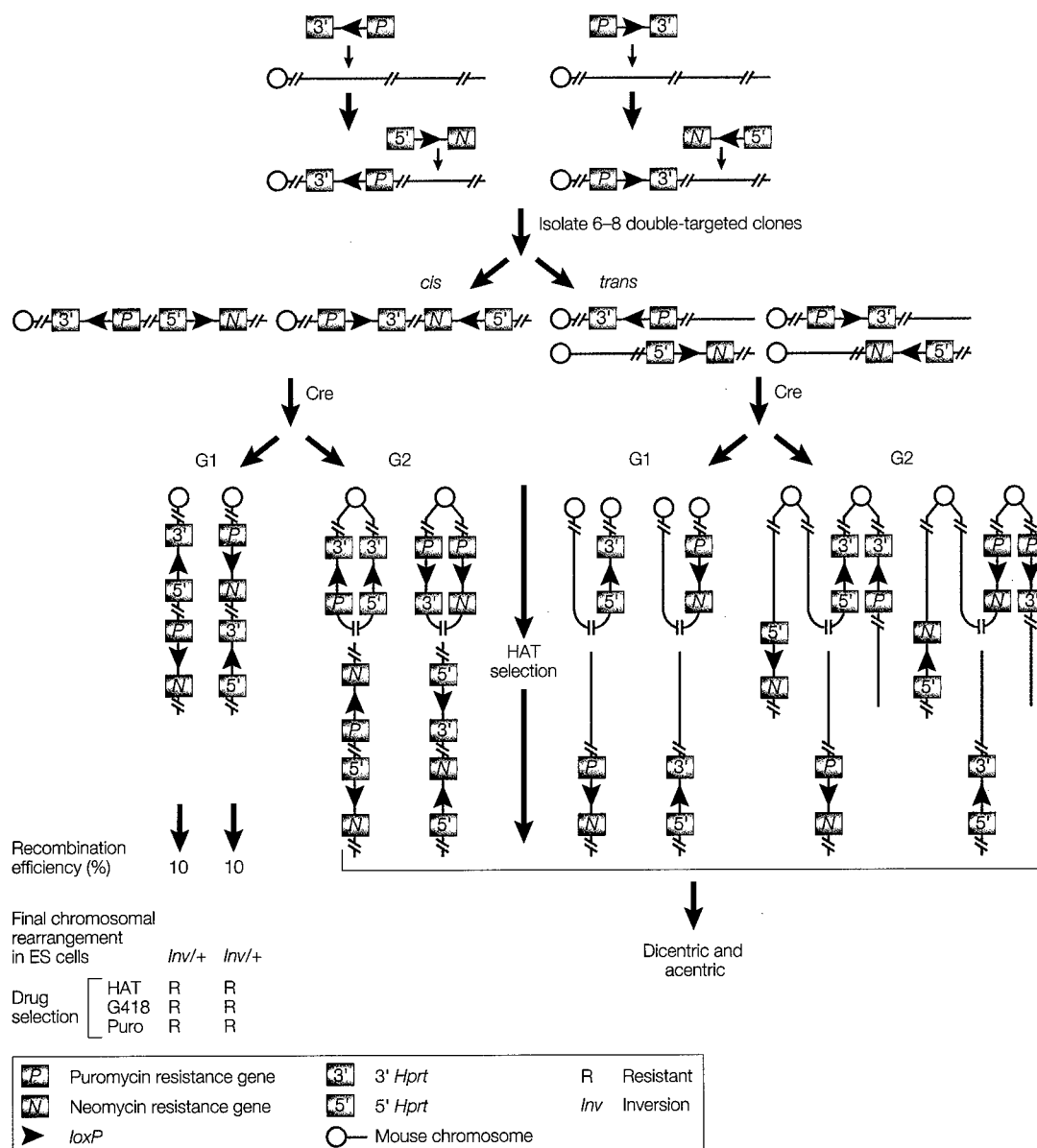

Figure 4 | **Engineering an inversion in embryonic stem cells.** An experimental procedure for engineering chromosomal inversions. G1 and G2 indicate the different phases of the cell cycle in which recombination occurs. Only orientations of the cassettes that result in inversions are illustrated. Cre-mediated recombination at G1 or G2 will result in various products. HAT, hypoxanthine, aminopterin and thymidine; Hprt, hypoxanthine phosphoribosyl transferase; Puro, puromycin.
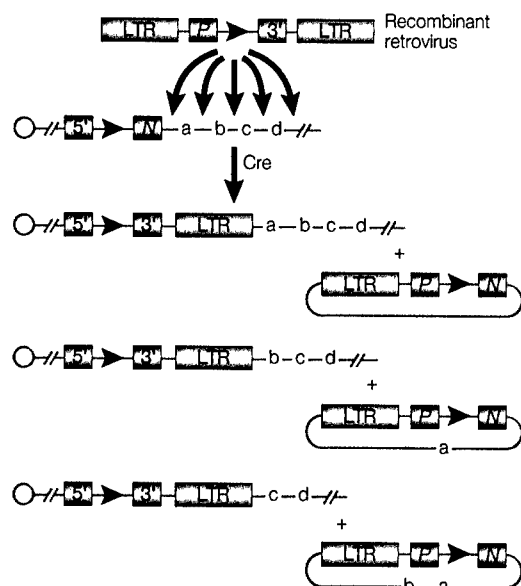
Figure 5 | **Nested chromosomal deletions induced with a retroviral vector.** The first deletion end point is fixed by targeting the 5′*Hprt* cassette and a *loxP* site to a predetermined locus. The 3′*Hprt* cassette and the second *loxP* site are then integrated randomly into the embryonic-stem-cell genome using a recombinant retroviral vector. For clarity, only G1 recombination events from retroviral orientations that result in deletions are illustrated. Cre catalyses recombination between the *loxP* sites (red arrowheads), and HAT medium is then used to select for the clones that carry the recombinant chromosomes. The nested deletions can be identified from a pool of HAT-resistant clones on the basis of their sensitivity to G418 and puromycin. 3′, 3′*Hprt*; 5′, 5′*Hprt*; a–d, genetic markers; HAT, hypoxanthine, aminopterin and thymidine; *Hprt*, hypoxanthine phosphoribosyl transferase; LTR, retroviral long terminal repeat; *N*, the neomycin resistance gene; *P*, the puromycin resistance gene. (Modified with permission from REF. 33.)

To induce a translocation *in vivo*, mice that carry both targeted end points can be crossed with transgenic mice that express *cre* under the control of a regulatory element with the desired tissue and temporal specificity[15,16]. This approach not only has been used to generate better models of human leukaemia-associated translocations, such as the t(8;21)(q22;q22) (REF. 15) and t(9;11)(p22;q23) (REF. 16) translocations, which cause acute leukaemia, but also circumvents the problem of transmitting translocations through the male germ line, as the presence of chromosomal translocations in male germ cells can cause infertility[40]. Recombination events that give rise to chromosomal translocations can reach recombination efficiencies of 10^{-4} to 10^{-6} in tissues that express Cre[15]. Although these recombination rates are low, they can mimic the rare genetic events that are crucial steps in neoplastic transformation (as discussed below).

Applications of chromosomal engineering

About 0.6% of all newborn human infants have cytogenetic imbalances[2], so chromosomal anomalies are a principal cause of human genetic disease. Somatic chromosomal translocations are crucial events in the formation of many types of human tumour, such as leukaemia, lymphoma and sarcoma[3]. Because there are many conserved linkage groups between the human and mouse genomes, the chromosomal rearrangements that are involved in human disease can be modelled in mice. These mouse models can be used to study the molecular events that are associated with these diseases. Mouse models of chromosomal deletions can also be used to analyse rearranged chromosomal regions and can facilitate the identification of the genes that are involved in the clinical features of chromosomal disorders.

The other main driving force behind recent advances in mouse chromosomal manipulation has been the need to generate resources to facilitate genetic screens[28]. Chromosomal rearrangements, such as visibly marked deletions and balancer chromosomes, have been instrumental in the success of genetic screens in *Drosophila*. In mice, mutagenesis efforts to generate and map recessive mutations have been hindered by the lack of marked deletions in most regions of the genome and by the unavailability of balancer chromosomes. The creation of these reagents through chromosomal engineering technology will change future strategies for large-scale, recessive genetic screens in mice and will facilitate the functional analysis of the mouse genome.

Modelling human disease. Among human chromosomal rearrangements, deletions constitute an important class. Deletions are often identified when haploinsufficient gene(s) in the deleted region cause a clinical phenotype. The positive-selection-based Cre/*loxP* strategy for engineering defined chromosomal deletions is uniquely suited for identifying and analysing mammalian haploinsufficient loci. Alternative strategies for generating deletions that use negative selection[24,30–32] and irradiation[35–37] suffer from the disadvantage that they do not generate selectable reciprocal products. However, the positive-selection-based strategy allows duplications to be recovered from *trans* recombination events (see supplementary Table 1 online). Duplications are important experimental tools because they allow haploinsufficient deletions to be maintained, and allow mice that harbour both a deletion and the reciprocal duplication to be recovered because they are genetically balanced.

Using chromosomal-engineering techniques, key genetic elements in several human chromosomal deletion disorders have recently been identified by engineering mouse chromosomal deletions in regions that are homologous with those deleted in certain human deletion disorders, such as DiGeorge syndrome and Prader–Willi syndrome[13,14,17,18]. The power of this approach has been illustrated by modelling the deletion that is involved in DiGeorge syndrome. DiGeorge syndrome is associated with a hemizygous deletion on human chromosome 22, del(22)(q11.2; q11.2). The DiGeorge region had been recalcitrant to molecular dissection in humans[41]. Despite intensive efforts,
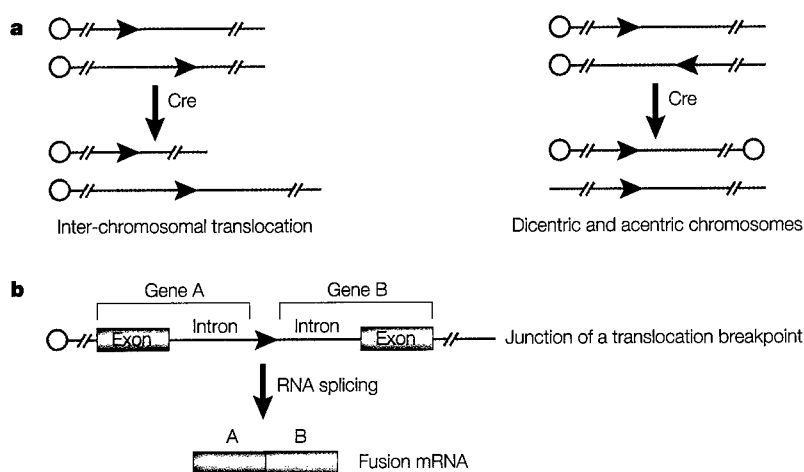
Figure 6 | **Engineering chromosomal translocations.** A strategy for engineering a chromosomal translocation and an associated fusion gene. **a** | Cre-mediated recombination leads to chromosomal translocation or dicentric and acentric chromosomes, depending on the relative orientations of the *loxP* sites (red arrowheads) on two non-homologous chromosomes. **b** | An in-frame fusion mRNA and protein can be generated by engineering an appropriate junction at the translocation breakpoint.

on mouse chromosomes 4 and 16, were used as the end points for Cre-mediated recombination. In t(9;11)(p22;q23), the translocation generates a fusion gene from the genes *MLL* (myeloid/lymphoid or mixed-lineage leukaemia) and *AF9* (myeloid/lymphoid or mixed-lineage leukaemia; translocated to, 3). To generate such a fusion gene in mice, the mouse *Af9* and *Mll* genes, located on chromosomes 4 and 9, respectively, were used as the rearrangement end points. Mice with double-targeted end points were crossed with transgenic lines that express *cre* in various organs, including the brain, and the desired rearrangements were produced in their progeny. However, *cre* expression has not yet been targeted to the haematopoietic cell lineages and, possibly as a result, leukaemia has not been reported in the mice that carry these translocations[15,16].

These translocations illustrate a further advantage of the Cre/*loxP* chromosomal engineering system. Inducing recombination *in vivo* can generate chromosomal deletions, duplications or translocations[15,16,25,43,44]. This approach can often be essential when the rearrangements cause ES-cell lethality[25] or embryonic death[26], or when modelling human chromosomal rearrangements that occur only in certain somatic cell types[15,16].

Engineered chromosomes for functional analysis.
Experimental approaches for the functional characterization of the genome of an organism rely on the generation of mutations. For the mutational analysis of diploid organisms, such as the mouse, genetic tools, such as marked deletions and inversions, are important reagents because they facilitate rapid genetic mapping and maintenance of randomly generated mutations, such as those generated during ethylnitrosourea (ENU) mutagenesis screens. The development of chromosomal engineering techniques has significantly expanded the repertoire of these powerful genetic tools.

In an effort to functionally analyse mouse chromosome 11, 18 deletions have been engineered on this chromosome using Cre/*loxP* technology[26,33] (Y.Y. and A.B., unpublished data; see also link to the Chromosome 11 deletion map). This work has generated mouse lines that carry regions of segmental haploidy, which can be used to screen ENU-mutagenized mice to identify recessive mutations. Smaller nested deletions can then be used for complementation testing, to narrow down the genomic location of a mutation as a prelude to cloning. Thereafter, the mutated gene can be identified by genomic complementation with BACs[45,46] and/or by sequencing the entire mutation-carrying region.

Although many deletions have now been generated on mouse chromosome 11, several of these deletions are haploinsufficient[26] (Y.Y. and A.B., unpublished data). Mice that carry these deletions either die during embryogenesis or show disease phenotypes. This prevents their use in genetic screens, although it does identify regions of the genome that are worthy

including the analysis of the finished genomic sequence of chromosome 22 (REF. 42), the gene(s) responsible for the clinical features of this disorder have not been identified using human molecular genetic approaches.

An alternative strategy exploiting chromosomal engineering was used to generate a 1.2-Mb deletion, Df(16)1, in the region of mouse chromosome 16 that corresponds to the minimal DiGeorge region on human chromosome 22 (REF. 13). The hemizygous deletion mice, Df(16)1/+, develop cardiovascular defects similar to those observed in DiGeorge syndrome patients. Importantly, in mice that harbour both Df(16)1 and the reciprocal duplication, no heart defects are detected, proving that reduced gene dosage in the deleted region is responsible for the mutant cardiovascular phenotype seen in the Df(16)1/+ mice[13]. To locate the gene(s) involved in this phenotype in Df(16)1, smaller overlapping deletions were generated by using known end points or by using randomly generated nested deletions that were induced by a recombinant retrovirus[33]. Mice that carry these sub-deletions were then analysed for heart defects. These studies narrowed down the candidate interval to a region that contained a few genes, one of which — *Tbx1* — went on to be identified as the haploinsufficient gene that causes the principal cardiovascular defects in DiGeorge syndrome[18]. The same conclusion was reached independently by a second group that also used chromosomal engineering techniques[17].

Progress has also been made in efforts to model human leukaemia-associated translocations, such as t(8;21)(q22;q22) (REF. 15) and t(9;11)(p22;q23) (REF. 16). In t(8;21), the breakpoints of the translocation are located in the genes *AML1* (acute myeloid leukaemia 1; also called *RUNX1*, runt-related transcription factor 1) and *ETO* (also called *CBFA2T1*, core-binding factor, alpha subunit 2; translocated to, 1). To model this translocation, the orthologues of these genes, located

ENU
(*N*-ethyl-*N*-nitrosourea). A potent mutagen that primarily generates single-base-pair mutations in mouse spermatogonia germ cells.

of further analysis. A deletion can be generated that encompasses a smaller interval as a way to avoid haploinsufficient gene(s), but this reduces the number of genes located in the interval. A mouse line that carries a smaller deletion is therefore not an efficient tool for conducting a genetic screen because the

number of recessive mutations that could be detected by using it would be significantly reduced. To overcome this problem, we have generated inversion chromosomes.

Using engineered inversions for mutagenesis screens has several advantages. First, unlike a deletion, a 20- to 30-cM inversion can be generated without causing a detrimental effect to mice. So, by using an inversion, a much larger genomic region can be screened. Second, a heterozygous inversion effectively suppresses crossing over in the inverted genomic segment because a single crossover between loci in the rearranged interval leads to inviable acentric and dicentric chromosomes or aneuploidy. Therefore, inversions can be used to maintain the genomic integrity of a mutagenized region. Third, an inversion can be designed to function as a balancer chromosome by tagging it with a recessive lethal mutation, which prevents animals that carry a homozygous inversion from being viable. Finally, a coat-colour marker can be added to an inversion chromosome so that its inheritance can be followed without requiring the genotypic analysis of progeny.

To facilitate the isolation of ENU-induced recessive mutations on mouse chromosome 11 (see link to Chromosome 11 ENU mutagenesis programme), the first mouse balancer chromosome was constructed on chromosome 11 using Cre/loxP-mediated recombination[20] (FIG. 7a). This balancer chromosome is based on a 24-cM inversion between the Trp53 gene and the Wnt3 gene. Mice that are homozygous for this inversion die during embryogenesis owing to the disruption of the Wnt3 gene, which is required for embryonic development[47], at one of the inversion end points. In addition, a coat-colour marker, K14–Agouti, has been inserted into the mutated Wnt3 locus. Such a marked balancer chromosome constitutes an ideal reagent for the isolation of novel ENU-induced recessive mutations in a three-generation breeding scheme[21] (FIG. 7b).

Conclusions and perspectives

ES-cell technology has drastically enhanced our ability to engineer various types of mouse genomic alteration, which now include single-gene knockouts, single-base-nucleotide alterations, conditional mutations (see review by Mark Lewandoski on p743 of this issue for more on this technique) and megabase rearrangements. Novel ES-cell-based technologies for genomic manipulation will undoubtedly continue to emerge.

The technologies of chromosome manipulation will become easier to apply as the mouse genome sequencing project progresses (see link to NCBI's mouse genome sequencing page). This is because the availability of a complete mouse genome sequence will facilitate the selection of end points and the construction of targeting vectors for use in engineering-defined chromosomal rearrangements. The mouse genome sequence will also help with identifying the location of random integration sites in nested chromosome deletions.
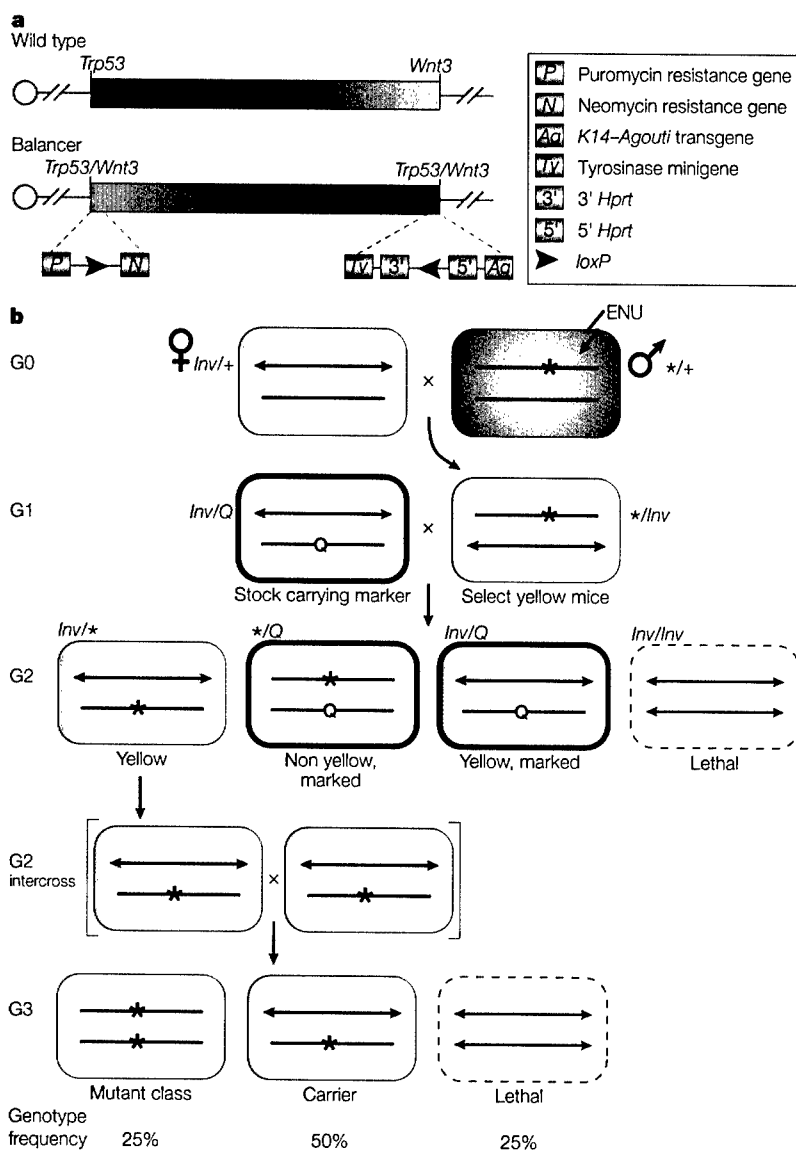

Figure 7 | **A mouse balancer chromosome and its use in ENU mutagenesis screens.**
a | The mouse balancer chromosome, Inv(11)8, is based on an inversion between Trp53 and Wnt3. Inv(11)8 mice carry an engineered coat-colour marker (agouti) and die when homozygous for the inversion during embryogenesis because of the targeted mutation at the Wnt3 locus. **b** | A breeding scheme to isolate recessive mutations using a balancer chromosome, such as Inv(11)8. In the first generation (G1), mice hemizygous for Inv(11)8 and an induced mutation are generated by mating ENU-treated males (*/+) to females that carry the balancer chromosome (Inv/+), which is marked by the dominant coat-colour marker, K14–Agouti. The G1 mice (*/Inv) are then crossed with mice that carry the balancer chromosome (Inv) and another visible marker (Q) on the homologous chromosome that is distinguishable from K14–Agouti. The Inv/* mice can be visually identified among the G2 mice, and Inv/Q and Q/* mice are not used further. Sibling matings between the Inv/* mice generate two classes of G3 mice, Inv/* and */*, which can be distinguished by the presence of K14–Agouti on the balancer chromosome. If all G3 animals carry K14–Agouti, the induced mutation causes embryonic lethality. Hprt, hypoxanthine phosphoribosyl transferase. (Modified with permission from REF. 21.)

The applications of these newly developed technologies are still in their infancy. Because they can now be generated in any region of the genome, marked deletions in mice, like their counterparts in *Drosophila*, will become invaluable for mapping genetic loci, such as quantitative trait loci[48]. Marked deletions and balancer chromosomes will also continue to gain importance in large-scale, recessive genetic screens in mice and will have a significant impact on efforts to functionally annotate the mouse genome. Studying DiGeorge syndrome in mice has illustrated the feasibility and benefits of using chromosomal engineering to generate models of human chromosomal rearrangements. Experiments are underway to engineer mouse models for other human congenital chromosomal disorders, such as Smith–Magenis syndrome (K. Walz and J. Lupski, personal communication) and trisomy 21 (REF. 49). The current mouse models of trisomy 21 are trisomic for only a portion of mouse chromosome 16, and mutant mice do not show all the

major clinical features of the disorder[9,50]. Because human chromosome 21 orthologues have been located to regions of conserved linkages on mouse chromosomes 10, 16 and 17, a better mouse model could be engineered that would carry segmental trisomies of all these genomic regions. Chromosomal engineering could also be used to generate small overlapping duplications in mouse chromosomal regions conserved with the trisomic human chromosome 21 regions to identify the crucial genomic domain(s) and causative gene(s) that are responsible for the clinical characteristics of the disorder.

Chromosomal-engineering technology has increased our ability to manipulate the mammalian genome, which has special significance in the current genomic era. The unique advantages of using this technology in the functional analysis of mammalian genomes and to develop animal models of human disease have been recognized and will continue to be shown in the coming years.

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Acknowledgements

🜨 Online links

DATABASES

The following terms in this article are linked online to:
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
AF9 | *AML1* | *ETO* | *MLL*
MGI: http://www.informatics.jax.org/
albino | *Hprt* | *Mll* | pink-eyed dilution | *Trp53* | *Wnt3*
OMIM: http://www.ncbi.nlm.nih.gov/Omim/
DiGeorge syndrome | Prader–Willi syndrome | Smith–Magenis syndrome | trisomy 21

FURTHER INFORMATION

Cell line Request Form:
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Chromosome 11 deletion map:
http://www.mouse-genome.bcm.tmc.edu/ChrEng/deletion.asp
Chromosome 11 ENU mutagenesis programme:
http://www.mouse-genome.bcm.tmc.edu/ENU/ENUHome.asp
Genetic and physical maps of the mouse genome:
http://carbon.wi.mit.edu:8000/cgi-bin/mouse/index#genetic
NCBI's mouse genome sequencing page:
http://www.ncbi.nlm.nih.gov/genome/seq/MmHome.html
STS Physical Map of the Mouse:
http://carbon.wi.mit.edu:8000/cgi-bin/mouse/index#phys
t(8;21)(q22:q22):
http://www.infobiogen.fr/services/chromcancer/Anomalies/t0821.htm
t(9;11)(p22;q23):
http://www.infobiogen.fr/services/chromcancer/Anomalies/t0911.html
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Introducing Defined Chromosomal Rearrangements into the Mouse Genome

Binhai Zheng,*,[1] Alea A. Mills,*,†,[2] and Allan Bradley*,†,[3]

*Department of Molecular and Human Genetics and †Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030

Chromosomal rearrangements have been instrumental in genetic studies in *Drosophila*. Visibly marked deficiencies (deletions) are used in mapping studies and region-specific mutagenesis screens by providing segmental haploidy required to uncover recessive mutations. Marked recessive lethal inversions are used as balancer chromosomes to maintain recessive lethal mutations and to maintain the integrity of mutagenized chromosomes. In mice, studies on series of radiation-induced deletions that surround several visible mutations have yielded invaluable functional genomic information in the regions analyzed. However, most regions of the mouse genome are not accessible to such analyses due to a lack of marked chromosomal rearrangements. Here we describe a method to generate defined chromosomal rearrangements using the Cre–*loxP* recombination system based on a published strategy [R. Ramirez-Solis, P. Liu, and A. Bradley, (1995) *Nature* **378**, 720–724]. Various types of rearrangements, such as deletions, duplications, inversions, and translocations, can be engineered using this strategy. Furthermore, the rearrangements can be visibly marked with coat color genes, providing essential reagents for large-scale recessive genetic screens in the mouse. The ability to generate marked chromosomal rearrangements will help to elevate the level of manipulative mouse genetics to that of *Drosophila* genetics.   © 2001 Academic Press

Gene targeting of individual genes in embryonic stem (ES) cells provides the means to assay gene

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[1] Current address: Department of Anatomy, University of California, San Francisco, CA 94143.

[2] Current address: Cold Spring Harbor Laboratory, P.O. Box 100, 1 Bungtown Road, Cold Spring Harbor, New York 11724.

[3] To whom correspondence should be addressed at Department of Molecular and Human Genetics/HHMI, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Fax: (713) 798-8142. E-mail: abradley@bcm.tmc.edu

function and to model human diseases in the mouse (1). Many human genetic disorders, however, are not associated with mutations in individual genes but with large chromosomal rearrangements such as translocations (1), deletions (2), duplications (3), inversions (4), and chromosome gain or loss (5). To faithfully model and to help elucidate the etiology of these human disorders, it is desirable to replicate the corresponding chromosomal abnormalities in the mouse. Such chromosomal rearrangements can recapitulate human disorders because of the existence of many regions of conserved synteny between the mouse and human genomes (6).

Because conventional gene targeting focuses only on a single genetic locus, the genetic changes that can be accomplished are quite modest in scale, usually below 20 kb (7). To engineer chromosomal rearrangements in ES cells, a system that can work at larger distances has to be adopted. Fortunately, two site-specific recombination systems have been shown to be functional in eukaryotic cells: the Flp–*FRT* system of the yeast 2-μm plasmid (8) and the Cre–*loxP* system from bacteriophage P1 (9). These systems are composed of recombinase proteins, Flp and Cre, which recognize and catalyze the recombination between specific DNA sequences, *FRT* and *loxP* sites, respectively. Both systems possess directionality in their sequence recognition, and recombination occurs only when the two sites juxtapose with each other in the same orientation. Gene targeting has been used mainly in conjunction with the Cre–*loxP* system to achieve tissue-specific knockouts and to generate subtle mutations (10).

Several groups independently worked out a Cre–

loxP-based strategy in conjunction with gene targeting in ES cells to generate large defined chromosomal rearrangements (chromosome engineering) (11–14). The principle is simple. By two consecutive gene targeting events one can deliver the *loxP* recombination sequence to two predefined loci in the genome. Expression of the Cre recombinase results in the desired rearrangement through site-specific recombination between the two *loxP* sites. Two groups generated specific translocations (12, 13). One group used this method to generate a deletion in a large gene (14), while our group generated multimegabase deletions, duplications, and inversions (11). Strategies for identifying recombinants varied: screening without selection (13), using negative selection (14), or using positive selection (11, 12). Selection is necessary when the rearrangement occurs at a low efficiency, which proved to be true in most cases. The method of positive selection, which is detailed below, is preferable to negative selection for two reasons. First, positive selection allows only clones that undergo a precise rearrangement to survive, ensuring specificity of the recombined products. In contrast, negative selection has a much higher background since a variety of other genetic changes in the cell that affect the selection cassette may enable clone survival. Second, positive selection allows for rearrangements without a net loss of genetic material (duplications, inversions, and translocations) to be readily engineered while negative selection is most directly applicable to deletions. Due to the obvious advantage of positive selection, in this article we focus on the experimental design and analytical tools for the positive selection-based chromosome engineering strategy. The two groups that independently developed the positive selection strategy used a virtually identical scheme (11, 12). We base our discussions on the vector system developed in our laboratory (11) but most conclusions can be extrapolated to the alternative vector system (12).

It should be stressed that in addition to producing animal models for certain human conditions, the ability to generate mice carrying engineered rearrangements, deletions, and inversions, in particular, has much wider applications in functional genomics and will have a profound impact on using the mouse as a genetic model organism. As detailed below, visibly marked deletions and balancer chromosomes can be engineered using this technology. Similar reagents have made *Drosophila* genetics possible.

Indeed, it is the potential to conduct large-scale mutagenesis with these deletions and inversions that is the driving force behind developing the technology of chromosome engineering in the mouse.

DESCRIPTION OF METHOD

General Strategy

The basis for the generation of chromosomal rearrangements in mouse ES cells is depicted in Fig. 1 (11). A positive selectable marker containing an
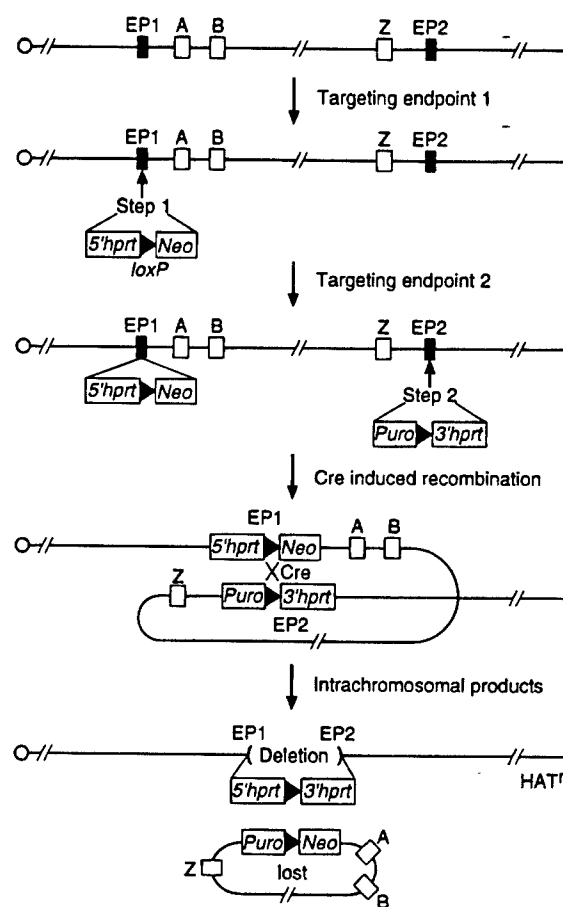

FIG. 1. Three-step Cre–*loxP*-based chromosome engineering strategy. EP1, endpoint 1; EP2, endpoint 2; A, B, and Z represent genetic loci between the two endpoints; *5'hprt* and *3'hprt*, 5' and 3' halves of the *Hprt* minigene; *Neo*, neomycin resistance gene; *Puro*, puromycin resistance gene. In step 1, a *loxP* site along with the *5'hprt* selection cassette is targeted to endpoint 1. In step 2, a *loxP* site along with the *3'hprt* selection cassette is targeted to the endpoint 2. In step 3, expression of the Cre recombinase leads to the desired rearrangement, in this case, a deletion.

intron, here an *Hprt* minigene, is divided into two nonfunctional fragments. Each fragment contains the 5′ or 3′ half of the minigene, respectively, and the two halves share an intron, which contains a *loxP* site in the same relative orientation. Either half of the *Hprt* minigene is nonfunctional. Recombination between the *loxP* sites by Cre, however, juxtaposes the two fragments, reconstituting the selectable marker and rendering the cell resistant to the selective drug HAT. To generate large chromosomal rearrangements, the two nonfunctional halves

of the selectable marker are delivered to distant sites in the genome, either on the same or different chromosomes, by two consecutive steps of conventional gene targeting (Fig. 1). For this purpose, either replacement or insertion vectors may be used (15). After expression of Cre, positive selection is used to isolate clones in which the selectable marker has been reconstituted.

The outcome of Cre-mediated recombination between the *loxP* sites will depend on the relative orientation and location of the two halves of the selectable
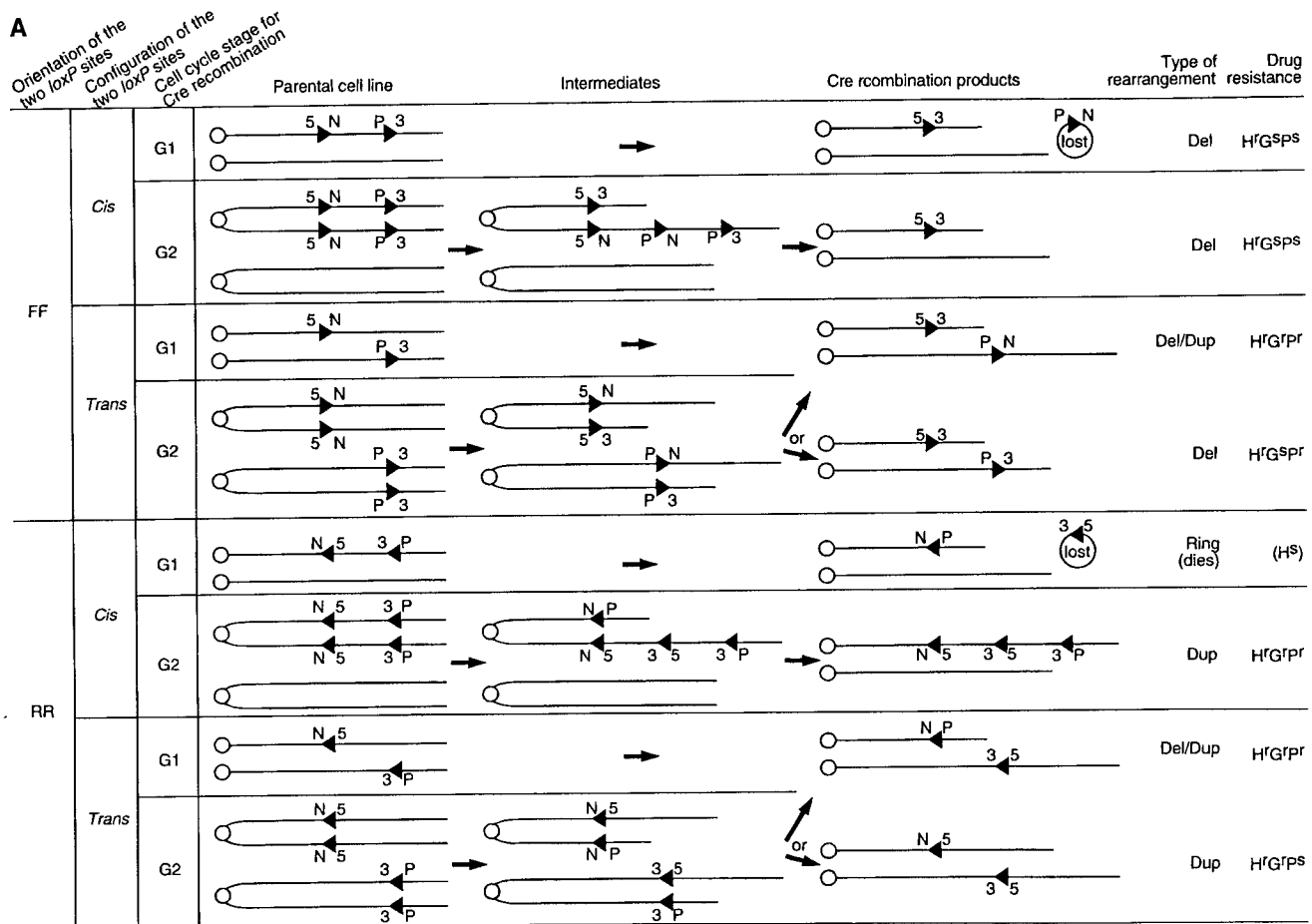
A

FIG. 2. Possible outcomes of Cre–*loxP*-mediated long-range recombination. (A) The two *loxP* sites are in the same orientation. (B) The two *loxP* sites are in opposite orientations. For G_2 events, only recombination between *loxP* sites on different chromatids is considered; G_2 recombination can also occur between two *loxP* sites on the same chromatid, but these events have the same consequence as the corresponding G_1 events and are therefore not shown. For each G_2 event, a recombination intermediate is shown. Note that using the strategy discussed in the text, only viable HAT-resistant products are recovered and scored. A *loxP* site is indicated by a solid triangle. Centromeres are indicated by circles. 5, *5′hprt*; 3, *3′hprt*; N, neomycin resistance gene; P, puromycin resistance gene; Del, deletion; Dup, duplication; Inv, inversion; Ring, ring chromosome; Dicen, dicentric chromosome; Acen, acentric fragment. In Drug Resistance column: H, HAT; G, G418 (neomycin); P, puromycin; superscript r, resistant; superscript s, sensitive. Another whole set of configurations where the *5′hprt* cassette is proximal to the telomere and the *3′hprt* cassette is distal are not shown, but the same logic applies and essentially the same types of recombination products are expected. Also see text for details.

marker relative to the centromere(s) and, in addition, the stage in the cell cycle this occurs. An extensive description of the outcome of the Cre–loxP recombination is depicted in Fig. 2, including deletions, duplications, and inversions resulting from Cre recombination between loxP sites targeted to the same chromosome or chromosome homologs. Translocations are omitted because they resemble the situation of deletion/duplications. A few concepts need to be clarified here. With respect to the orientation of the two loxP sites relative to each other, FF, or forward–forward, refers to the situation where the two loxP sites are in the same orientation with respect to the chromosome and both the 5'hprt and 3'hprt genes are outside of the loxP-flanked (floxed) region; RR, or reverse–reverse, refers to the situation where the two loxP sites are in the same orientation but both half hprt genes are inside the floxed region. Both FR and RF have opposite loxP site orientations, with the

5'hprt outside of the floxed region in FR but 3'hprt outside of the floxed region in RF. cis refers to the situation where the two loxP sites are on the same chromosome. trans refers to the situation where the two loxP sites are on the two chromosome homologs. If Cre recombination occurs before DNA synthesis in a cell cycle, the event is referred to as a G_1 event. In contrast, a G_2 event refers to one after DNA synthesis. In G_2, Cre recombination may occur between sister or nonsister chromatids. In any event, because of the positive selection applied, only clones that contain a reconstituted Hprt gene are recovered and scored.

In general, if the two loxP sites are located on the same chromosome or on each homolog of an autosome, inversions, deletions, or duplications will take place depending on the relative orientation of the two loxP sites. FF leads to deletion when in cis and deletion/duplication when in trans, while RR
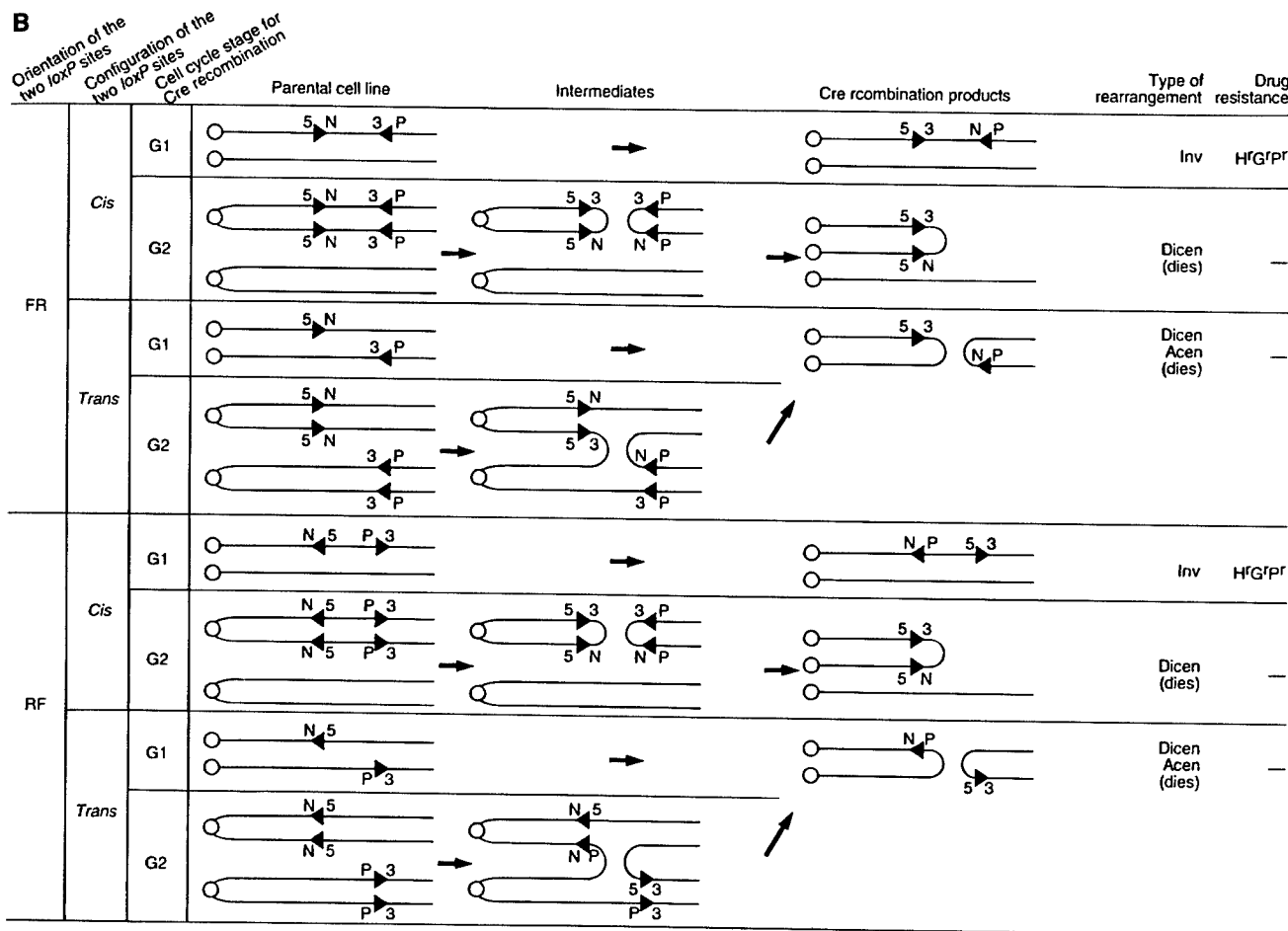

FIG. 2—Continued

leads to duplication when in *cis* and deletion/duplication when in *trans* (Fig. 2A). Both FR and RF lead to inversion when in *cis* and inviable dicentric and acentric chromosomes when in *trans* (Fig. 2B). On the other hand, if the two *loxP* sites (along with the relevant markers) are located on two nonhomologous chromosomes but in the same orientation relative to the centromeres, a chromosomal translocation will take place (not shown).

A panel of deletions across the genome will be a valuable resource for mouse genetics. In our laboratory, a massive effort to generate series of deletions along chromosomes (Chrs) 4 and 11 has been initiated. Chr 11 was chosen as it is extensively conserved with human Chr 17. Almost every gene mapped to human Chr 17 has a homolog on mouse Chr 11. Consequently, functional genomic information derived from mouse Chr 11 can be readily extrapolated to human Chr 17 (16). In addition, mouse Chr 11 is gene rich and carries several potential tumor suppressor genes (17, 18). Chr 4 was chosen because it has also been implicated in tumorigenesis by a number of loss-of-heterozygosity studies (19). The sizes of the deletions that we have sought to establish are usually a few centimorgans (cM). Although deletions of more than 10 cM have been made in the mouse by irradiation, the maximum size of a deletion that is compatible with normal embryonic development is probably region-dependent as two nonoverlapping deletions of 3–4 cM around *Hsd17b1* on Chr 11 are heterozygous lethal (20). The simple sequence length polymorphic (SSLP) microsatellite markers (21) are convenient deletion endpoints because of their well-mapped chromosomal locations. However, genes that have been mapped relatively accurately can also serve as endpoints, with the advantage that single-gene mutations are generated as well as chromosomal rearrangements.

Until the mouse genome is sequenced at high fidelity, for a given gene or microsatellite marker, the orientation of the genomic sequence (or transcript orientation for genes) relative to the centromere is generally unknown. Because the types of rearrangements made with the Cre-*loxP* recombination system depend on the relative orientation as well as position of the *loxP* sites on the chromosome (Fig. 2), the lack of such information necessitates the targeting of at least one endpoint with both *loxP* site orientations to ensure the ability to generate a specific rearrangement (deletion/duplication or inversion).

A general strategy to start a chromosome engineering experiment is as follows: (1) Target one endpoint with one orientation of *loxP* site. (2) Target the second endpoint with both *loxP* site orientations independently in the cell line already targeted at the first endpoint. (3) Perform Cre expression and analyses of recombination products using methods described below. One of the two orientations will be FR or RF so that an inversion can be obtained. The other orientation will therefore be either FF or RR where deletion/duplications can be generated from the *trans* events and deletions or duplications from the *cis* events. The availability of the mouse genome sequence information will simplify the design of the chromosome engineering experiment. It is necessary to generate multiple double-targeted cell lines (usually six to eight) for each combination of *loxP* site orientations to ensure that both *cis* and *trans* configurations are represented in the collection of double-targeted clones.

Analysis of HAT-Resistant Cre-*loxP* Recombination Products

There are five complementary techniques to analyze the HAT-resistant Cre recombination products: Cre recombination efficiency, selectable marker retention, junction fragment analysis by polymerase chain reaction (PCR), junction fragment analysis by Southern blot, and fluorescence *in situ* hybridization (FISH).

1. Cre Recombination Efficiency

The efficiency with which Cre catalyzes site-specific recombination depends on whether the two *loxP* sites are on the same chromosome and, if so, the distance between them. This can be conveniently measured as the recombination efficiency in an *in vitro* transient transfection assay. Here we define Cre recombination efficiency as the ratio of the number of HAT-resistant colonies to the number of colonies arising without drug selection after electroporation with the Cre expression plasmid. This ratio is a relative measure of efficiency as different Cre expression plasmids may result in different levels of Cre activity on the same substrate. The relative Cre recombination efficiency is very helpful in distinguishing among different recombination products. To provide a guideline for this efficiency, we have tested several genetic intervals ranging between 2 and 60 cM (21a). The general conclusion from these analyses is that Cre recombination efficiency on *cis* substrate decreases over increasing genetic distance (Fig. 3).

When the distance is small, *cis* deletions, *cis* duplications, and *cis* inversions occur at a similarly high Cre recombination efficiency. Within a few centimorgans, deletions can occur at an efficiency as high as 10^{-1}, comparable to the efficiency when *loxP* sites are just a few kilobases apart. Therefore, for this size range one needs to dilute the electroporated cells or transfect small quantities of Cre expression plasmid before plating to be able to pick single colonies. This high Cre recombination efficiency (tested for a 2-cM substrate) was realized after correction of a mutation in the original *3'hprt* cassette (21a).

trans recombination to generate deletion/duplications has a moderately low efficiency (about two to three orders of magnitude lower than *cis* for short distances), as expected for intermolecular recombination. If the *loxP* sites are in opposite orientations on different chromosomal homologs no viable recombination products are generated regardless of the distance between the *loxP* sites. Taken together, for short distances, if HAT-resistant colonies are not generated this indicates that the *loxP* sites are in opposite orientations in *trans*. If HAT-resistant colonies are generated at high efficiency this indicates recombination has occurred in *cis* to generate deletions, duplications, or inversions. The number of HAT-resistant colonies for *trans* deletion/duplications falls somewhere between these values. To distinguish between simple deletions and simple duplications, other tests such as drug resistance tests need to be performed (see below).

The situation is quite different when the distances

FIG. 3. Efficiency of Cre-mediated recombination over genetic distance. Percentage of Cre recombination efficiency (*Y* axis, in log scale) is plotted against genetic distance (*X* axis, in linear scale). The first data point represents a deletion. The other three data points with larger genetic distances represent inversions. Error bars indicate standard deviations. Reprinted with permission from *Molecular and Cellular Biology,* B. Zheng, M. Sage, E. A. Sheppeard, V. Jurecic, and A. Bradley, 2000, **20,** 648–655.

between the two *loxP* sites are large (more than 5 to 10 cM; the cutoff range has not been determined). Based on our work on Chr 11, large deletions (e.g., a 22-cM deletion on the distal part of Chr 11) may cause ES cell lethality or a severe growth disadvantage (21a). Such lethality may be due to a gene dosage effect of one or multiple genes within the deletion interval, a situation of haplo insufficiency. As a result, only cells that have gained an additional wild-type chromosome will survive. In these cases the assessed Cre recombination efficiency will depend both on the recombination efficiency and on the frequency of chromosome duplication (e.g., by nondisjunction). Consequently the apparent *cis* deletion efficiency for large distances could be very low. In such circumstances, the highest Cre recombination efficiency is a property of the class of clones that give inversions. Colonies are still inviable when the *loxP* sites are in opposite orientations in *trans*. Two groups of clones yield similarly low recombination efficiencies: *cis* deletions or *trans* deletion/duplication.

2. Selectable Marker Test

The second step in analyzing the Cre recombination products is to score for the presence or absence of the selectable markers. This takes advantage of the design of chromosome engineering cassettes (Fig. 1). In the targeting vectors for the endpoints, the selectable marker for gene targeting (*Neo*[r] or *Puro*[r]) and the half *hprt* gene (*5'hprt* or *3'hprt*) are on opposite sides of the *loxP* site so that they are always separated from each other after Cre recombination. This forms the basis for marker analysis.

The expected pattern of drug resistance of recombination products can be found in Fig. 2. As a rule, when two *loxP* sites are in the same orientation relative to the centromere, Cre recombination leads to deletions and/or duplications. If the two *loxP* sites are in *cis* with an FF orientation, Cre recombination leads to a simple deletion chromosome that is HAT-resistant but G418- and puromycin-sensitive. *trans* recombination with an FF orientation in G_1 leads to a deletion and a duplication. Since the recombination products are not segregated, the HAT-resistant cells are also G418- and puromycin-resistant. A deletion and a duplication can also result from a *trans* event involving nonsister chromatids in G_2 but the deletion may cosegregate with either the duplication chromosome or the single-targeted chromosome. While in the former case the product will be resistant to both G418 and puromycin, in the latter it will be puromycin-resistant but G418-sensitive. If the two *loxP* sites
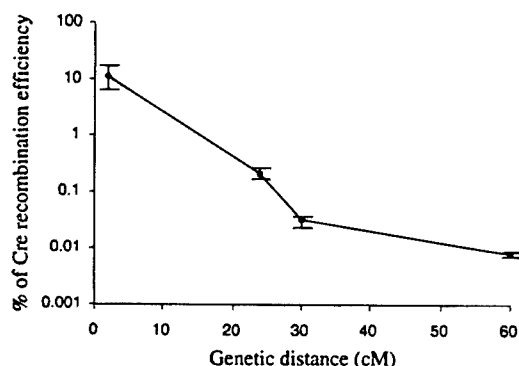
are in *cis* with an RR orientation, Cre recombination in G_1 leads to a deletion but it is HAT-sensitive and therefore will not be recovered. In this configuration, Cre recombination in G_2 between sister chromatids will yield a duplication that is HAT-resistant and will segregate with a wild-type nonsister chromatid. The cells carrying the reciprocal sister chromatid with a deletion will be lost due to HAT sensitivity. *trans* recombination with an RR orientation is analogous to the FF orientation described above.

The situation is much simpler when the two *loxP* sites are in opposite orientations relative to centromere (FR or RF). When the two *loxP* sites are in *cis*, Cre recombination leads to inversions that will be resistant to neomycin and puromycin. However, Cre-mediated recombination between reversely oriented *loxP* sites on two sister chromatids in G_2 leads to inviable dicentric (or acentric) chromosomes. When the two *loxP* sites are in *trans*, Cre recombination also leads to dicentric and acentric chromosomes.

The selectable marker tests do not always give definitive results. Sometimes cells exhibit partial resistance (or partial sensitivity). These may be due to the presence of sister cells with reciprocal recombination products that are rescued by cross-feeding, a phenomenon commonly observed with ES cell cultures. Generally, the faster-killing drugs give less ambiguous resistance test results. For instance, the puromycin test is usually more reliable than G418 resistance. In general, it is advisable to perform control plating experiments at relatively low cell densities with all three selection drugs to ascertain if the resistance to a selection drug is caused by contaminating cells or is a property of the HAT-resistant clones. It is also possible to determine the existence of selectable markers by Southern blots using the marker gene as a probe.

3. Junction Fragment Analysis by PCR and Sequence Analysis

To determine if a precise rearrangement has occurred, one can amplify the recombination junction fragment by PCR that is unique to the rearranged allele (Fig. 4A). The PCR products can be sequenced to determine the precision of the rearrangement at the single-nucleotide level. However, all rearrangements that we have analyzed so far by PCR and sequencing proved to be precise rearrangements. Therefore, this step is more of a precautionary step in analyzing a recombination product. The PCR strategy can also be used to genotype the mice when rearrangement products are transmitted through

germ line. Note, however, that the PCR in Fig. 4A is universal and will not distinguish among different rearrangements.

4. Junction Fragment Analysis by Southern Analysis

To determine more conclusively if a rearrangement has occurred, we routinely use Southern blots to analyze the recombination products. Figure 4B illustrates the scheme for such an analysis. The advantage of Southern analysis over PCR is that one can assess a larger junction region. Because the breakpoints have been confirmed to be targeted in these experiments; a probe internal to the homology region can be used as well as the external probes used for gene targeting. It is also desirable to analyze junctions at both break points. When analyzing simple deletions, one can use probes internal to the deletion to confirm the absence of the targeted fragment in the deletion.
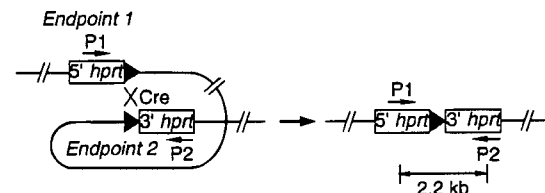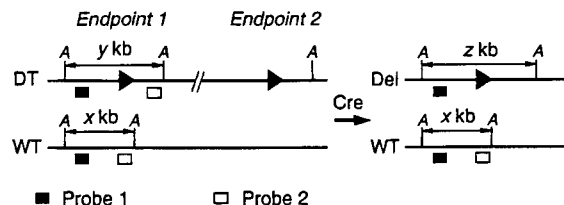

FIG. 4. Schematic of PCR and Southern analysis of chromosomal rearrangements. (A) PCR analysis. P1, primer 1; P2, primer 2. Because the two endpoints are megabases away from each other on the same chromosome (as shown) or on different chromosomes, a PCR product can be detected only after Cre-mediated recombination. (B) Southern analysis. WT, wild type; DT, double targeted; Del, deletion; A, restriction enzyme A. Probe 1 lies outside of the deletion at endpoint 1 and detects restriction fragments of different sizes: x kb for wild type (WT), y kb for the targeted allele (DT) and z kb for the deletion allele (Del). Probe 2 lies inside of the deletion at endpoint 1 and detects unique wild-type and targeted alleles but not the deletion allele. In the case of deletion/duplication, inversion, or translocation, probe 2 can detect a unique fragment for the rearranged allele. In addition to probes at endpoint 1, similar probes can be developed at endpoint 2.

5. FISH Analysis of the Recombination Products

Fluorescence *in situ* hybridization is a very effective method to analyze rearrangements induced by Cre–*loxP*, especially for larger rearrangements. To detect an inversion, two probes internal to the inversion must be differentially labeled and used for hybridization. A change in the order of the two probes relative to the centromere indicates an inversion. It is preferable to include a third probe that lies exter-
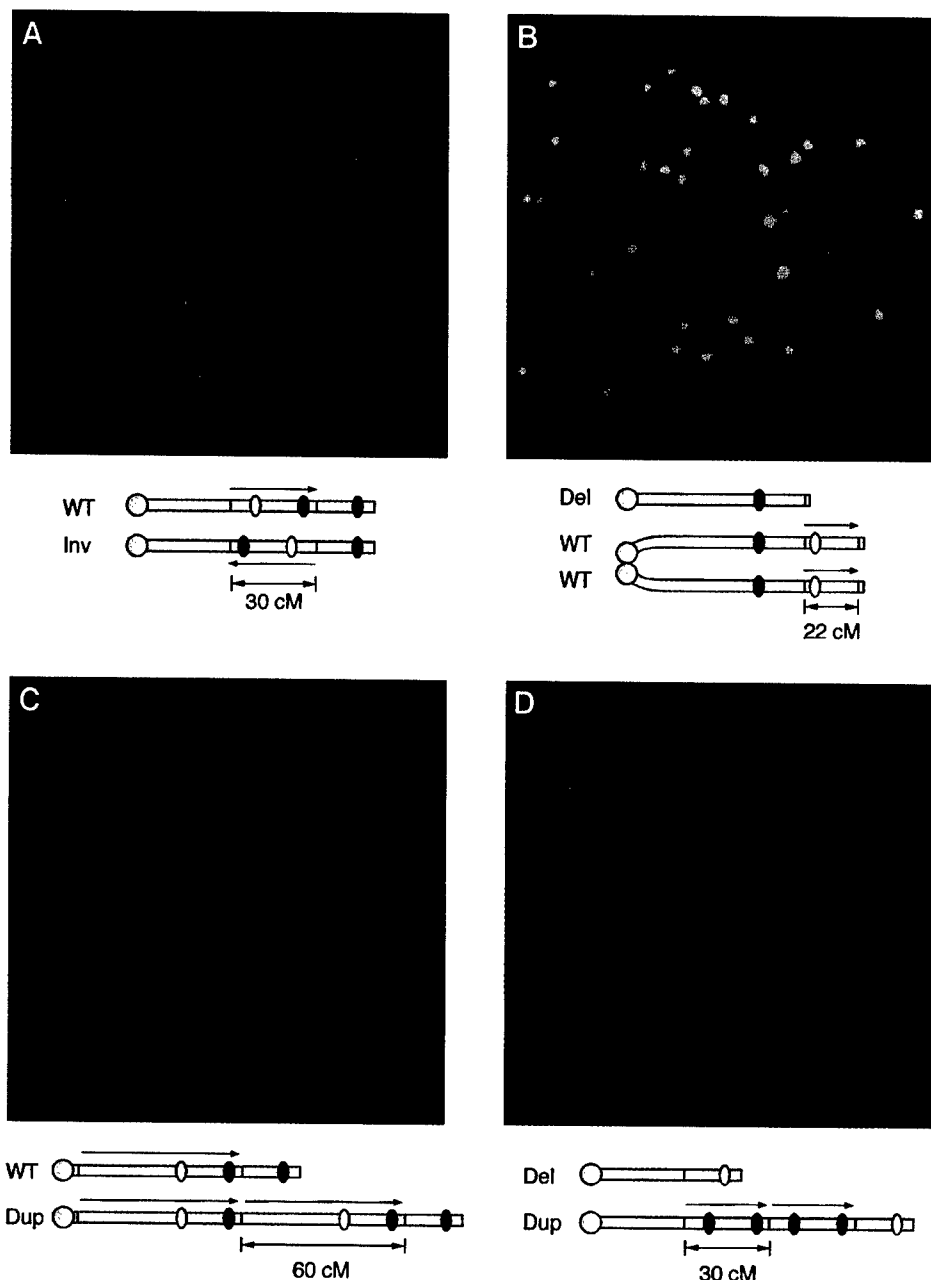

FIG. 5. FISH analysis of chromosomal rearrangements. (A) A 30-cM inversion between *D11Mit142* and *Hsd17b1*. (B) A 22-cM deletion between *Hsd17b1* and *D11Mit69*. This deletion causes heterozygous ES cell lethality so that only cells that have gained an additional wild-type chromosomes will survive, as indicated by the presence of two wild-type chromosomes that exist in an isochromosome (Robertsonian) configuration in this particular cell line. (C) A 60-cM duplication between *D11Mit71* and *Hsd17b1*. (D) A balanced 30-cM deletion and duplication between *D11Mit142* and *Hsd17b1*. Two or three probes are differentially labeled and artificially colored.

nal to the inversion as a control, both as an additional reference in determining the relative order of the two internal probes and as a way to detect any additional gross change in the chromosome of interest that may be associated with the Cre–loxP-induced rearrangement (e.g., trisomy). Such a control probe becomes necessary if the inversion is small (less than a few centimorgans), in which case the analysis of interphase nuclei is required. To detect duplications and deletions, one probe internal to the interval (test probe) and one probe external to the interval (control probe) need to be used. A deletion is simply indicated by the absence of the test probe on one chromosome. A duplication is indicated by duplicated signals along the chromosome. Note, however, that these duplicated signals should be distinguished from (and are on top of) the pair of signals for each probe from the two chromatids of each metaphase chromosome. That is, four signals (in two pairs) are expected for a probe within the duplicated region. Duplications can be easily detected if they are large (e.g., ≥ 10 cM). However, if the duplications are smaller than a few centimorgans, the duplicated signals may be recognized as a single dot on metaphase spreads.

FIG. 6. Coat color tagging of ES cell-derived mice. (A–C) Mice with a targeted *K14-Agouti* transgene (filled arrow) and their wild-type littermates (open arrow). (A) *p63*-targeted allele 1; (B) *p63*-targeted allele 2; (C) *Wnt3*-targeted allele. (D) A *Tyrosinase* transgenic mouse (filled arrow) with its wild-type littermate (open arrow).

Interphase nuclei must therefore be analyzed. For translocations between nonhomologous chromosomes, at least two probes, one proximal to the breakpoint on one chromosome and the other distal to the breakpoint on the second chromosome, should be used for analysis. Examples of FISH analysis on several rearrangements are shown in Fig. 5.

Marking Chromosomal Rearrangements with Coat Color Genes

A very useful feature of chromosomal rearrangements in *Drosophila* is that many of these are marked with a dominant visible marker (22). Such markers enable a chromosome to be followed without selection or genotyping, an indispensable innovation in *Drosophila* genetics. In mice, such a feature is also highly desirable if one would like to use the rearrangements in genetic screens. We therefore introduced and evaluated two coat color genes, developed previously in transgenic studies (23, 24), as visible markers for targeted alleles in ES cell derived mice. *K14-Agouti*, an *Agouti* cDNA sequence under the control of the *Keratin-14* gene promoter, had been shown to give a yellowing (or lightening) of coat color in an otherwise wild-type agouti mouse, with its expression dominant over wild-type agouti (23). The *Tyrosinase* minigene has been shown to give pigment to an otherwise albino mouse (24).

We tested the *K14-Agouti* marker at several chromosomal loci, such as *p63* (25), *Wnt3, D4Mit51, D4Mit70,* and *D11Mit142* (26) (and our unpublished data). Expression of the *K14-Agouti* at *p63*, a *Trp53* (also known as *p53*) homolog, is most dramatic, as two different alleles of *p63* targeted with the *K14-AgoutiK1* gene express a butterscotch or yellowing of the coat color (Figs. 6A, 6B) (26). The high level of expression of *K14-Agouti* at *p63* is not surprising as *p63* is expressed in the skin (25) and Agouti acts in a paracrine fashion (27). Most other loci tested, including *Wnt3, D4Mit51, D4Mit70,* and *D11Mit142*, show a lightening of the tail and back of earlobes without an apparent effect on body fur color (Fig. 6C) (26). As ectopic expression of Agouti may cause obesity (28–30), the low but detectable expression of this transgene may be advantageous. We also tested the *Tyrosinase* minigene at several loci including *D11Mit69, p63* and *Trp53.* The expression of the *Tyrosinase* minigene is not detectable at *D11Mit69* (assessed in an albino background) (26). At the *p63* locus, it is not expressed in the coat but a moderate
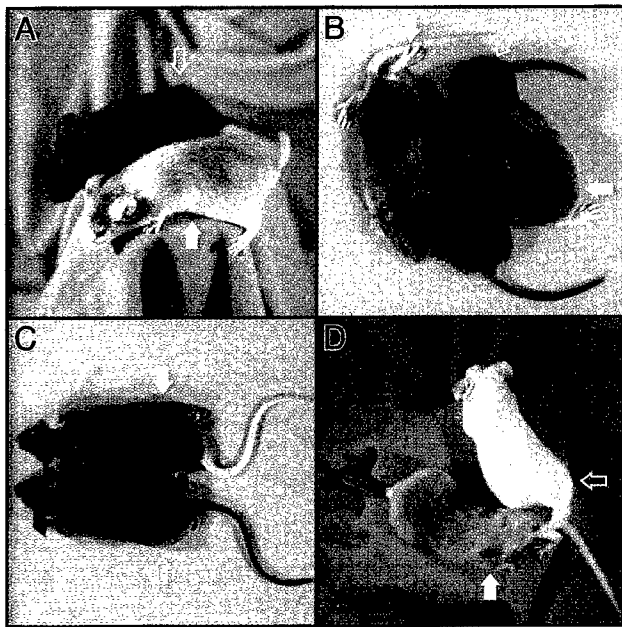
darkening of the eye was observed in the newborns (our preliminary data). At the *Trp53* locus, expression of the *Tyrosinase* minigene gives a grayish coat color (our preliminary data). However, the *Tyrosinase* minigene is not expressed on an inversion (see below) where it has been transferred as an endpoint from the *Trp53* locus to the *Wnt3* locus 24 cM away (31).

The results from these studies with ES cell-derived mice are consistent with our study with transgenic mice. In that study, three of three transgenic *K14-Agouti* founders had a yellowing of the coat color. However, there is some variability in penetrance and expressivity in the phenotype, as has been observed in other transgenic studies. In studies with the *Tyrosinase* minigene, only 3 of 12 transgenic founders showed a gray or light gray coat color in an albino background (Fig. 6D). Therefore, *K14-Agouti* is an effective marker for visual genotyping of targeted alleles while the *Tyrosinase* minigene is more position dependent as a genetic marker. Development of additional genetic markers that have no or minimum side effects will be extremely rewarding for manipulative mouse genetics. One way to approach this is to improve the *Tyrosinase* minigene by optimizing the promoter to direct high levels of expression in melanocytes. The availability of multiple independent markers that are not in the same genetic pathway will greatly enhance our ability to design sophisticated genetic screening procedures in mice, similar to those that have been enjoyed by the *Drosophila* genetics community for years.

To illustrate the use of a coat color marked chromosomal rearrangement, we generated a balancer chromosome on mouse Chr 11 (31). Balancer chromosomes are recessive lethal, dominantly marked inversions that suppress crossovers. They are used to maintain lethal mutations and to maintain the integrity of the mutagenized chromosomes in genetic screens in *Drosophila* (22, 32). Following the *Drosophila* paradigm, we constructed a 24-cM recessive lethal, dominantly marked inversion between *Trp53* and *Wnt3*. *Wnt3* was chosen as an endpoint because a *Wnt3* mutation confers homozygous lethality (33). The genetic distance (24 cM) was chosen to be sufficiently large to be useful for a significant part of Chr 11 (24 cM/80 cM $\cong$ 30%) while sufficiently small to minimize double crossovers that obviate the balancing effect. Both endpoints were targeted in such a way as to inactivate the endpoint genes. The *K14-Agouti* transgene was introduced to the *Wnt3* locus during gene targeting. Similarly, the *Tyrosinase* gene

was introduced to the *Trp53* locus. The inversion was confirmed by FISH analysis and transmitted through the germ line. The *K14-Agouti* gene tags the inversion mice with a lightening of the tail and ear color in a nonalbino background, although the *Tyrosinase* minigene is not expressed on the inversion. This inversion proved to suppress recombination within the inversion interval (no recombination out of 108 meioses examined). It therefore fulfills the three criteria set for a balancer chromosome: suppressing crossovers, dominantly marked, and recessive lethal. This inversion can be used to maintain lethal, sterile, or other detrimental mutations within the 24-cM interval, including targeted alleles at the *Brca1* or *HoxB* series. More importantly, this chromosome can be used for large-scale recessive genetic screens targeted for the 24-cM region on Chr 11 by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis (34). In the future, it is expected that a series of balancer chromosomes will be constructed across the mouse genome using the approach described above. Because each balancer chromosome can span about one third of a chromosome, a limited number of inversions ($\sim$ 60) can cover the entire mouse genome. If a balancer chromosome for an entire chromosome is desired, one can construct multiple (or compound) inversions by a variety of approaches, such as using mutant *loxP* sites (35, 36), using a different site-specific recombination system (37, 38), or re-targeting to delete the preexisting *loxP* sites before constructing the next inversion.

Libraries of Targeting Vectors for Chromosome Engineering

A deletion panel across the mouse genome will be useful resource for mapping studies and mutagenesis programs. One rate-limiting step of engineering chromosome rearrangements in mice, particularly in large-scale experiments, is the molecular cloning required to build individual targeting vectors. To streamline this process, we developed a two-library system for large-scale chromosome engineering (Fig. 7) (26). The unique feature of these libraries is that once a clone is isolated, it is essentially ready to be used for insertional targeting in ES cells. The vector backbones of the two libraries each bear a complementary set of genetic markers tailored both for Cre-*loxP* based chromosome engineering and for single gene knockouts. The vectors additionally carry coat color genes so that the rearrangements (or targeted mutations) can be visibly marked. Because only one

contiguous random genomic insert can be cloned into a vector to construct a library, insertional targeting is used instead of the more widely used replacement vectors. Insertion vectors can be mutagenic if the genomic insert contains the midportion but lacks both the 5' and 3' parts of the gene of interest. For instance, two *p63* null alleles have been generated with two clones independently isolated from the *3'hprt* library (25). Although there are some limitations for single-gene knockouts, vectors from the libraries are ideal for chromosome engineering purposes. The availability of these libraries makes it feasible to rapidly generate panels of marked deletions across the genome.

Germline Transmission

Certain deletions may cause embryonic lethality in heterozygotes, even when the deletions are relatively small (a few centimorgans) (20). This deletion-associated lethality is probably region-specific and may be due to a dosage effect (haplo insufficiency) of one or multiple genes. In addition, even when a small deletion is heterozygous viable (which is expected for most regions of the mouse genome), the deletion may be associated with reduced fitness such as fertility. Therefore, chimeric mice are best produced by injecting deletion/duplication ES cells resulting from Cre recombination between two *loxP* sites *in trans*
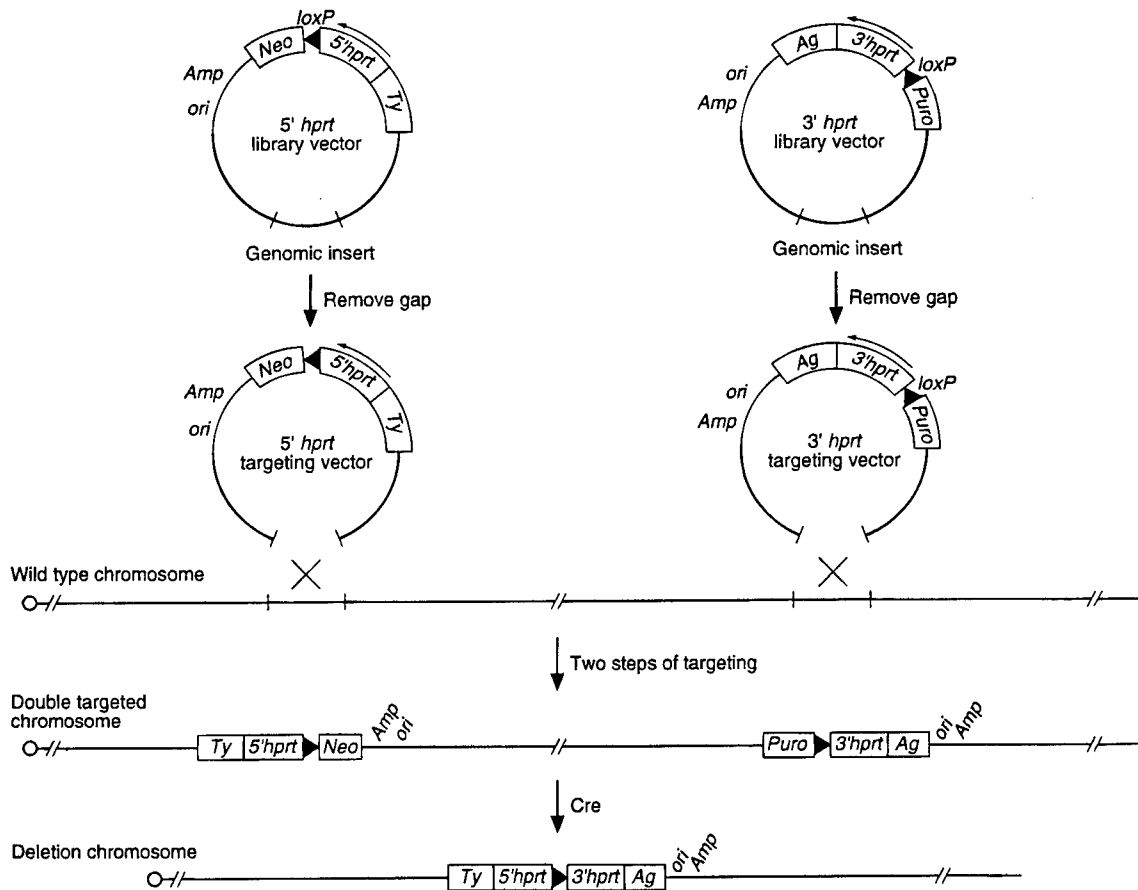

FIG. 7. Using vectors from the 5' and 3' half targeting vector libraries to generate deletion panels. The two deletion endpoints are isolated from the two libraries respectively. A gap is then created in each vector to develop a probe for detecting targeted events by Southern analysis. The gapped vectors are then used for targeting the endpoints. Genomic inserts in the vectors can be easily flipped by one step of subcloning using two flanking rare cutting sites (not shown) to ensure that each endpoint can be targeted with two different orientations of the *loxP* sites that are required to generate different types of rearrangements. Note that using this vector system, only FF configuration (discussed in the text) will give a coat color-tagged deletion on Cre-mediated recombination.

in the same orientation. This strategy also allows for the production of both deletion and duplication mice. This becomes absolutely necessary when the deletion is heterozygous lethal so that deletion mice can only be produced and maintained in a duplication background.

Another cause for deletion-associated heterozygous lethality is imprinting. This is usually a problem only when the gene(s) involved is paternally expressed (maternally imprinted). Since ES cells are usually XY in origin, a maternally expressed gene inherited from a male chimera is not expressed anyway. In contrast, when the gene is paternally expressed, the deletion progeny will be a functional null for this gene. If the imprinted gene is essential for normal development, the deletion progeny cannot survive. This problem can be circumvented by the use of XX ES cells. Alternatively, if the deletion size is small, one may generate the deletion by breeding mice targeted at both anchor points to a universal deletor line (39–41).

Tissue-Specific Chromosome Engineering

As discussed above, heterozygous chromosomal deletions may cause embryonic lethality. This is expected for most large deletions covering a significant part of any chromosome and is consistent with the notion that no monosomy in the mouse is compatible with embryonic development to term. Some small deletions will also be lethal and this lethality is probably chromosome region specific. Therefore, to study a specific biological problem, such as tumorigenesis in certain tissue/cell types, it is desirable to generate deletions in a tissue/cell type-specific manner. As the efficiency of Cre recombination is of the order of 10^{-1} to 10^{-2} for substrates of a few centimorgans, these rearrangements can be engineered somatically. An important use of this strategy is in modeling somatic loss of heterozygosity in tumorigenesis. Such an approach can be potentially used to identify novel tumor suppressor genes, especially for genes mutated in sporadic cancers, in conjunction with an insertional mutagen (e.g., a retrovirus) (Fig. 8). For other
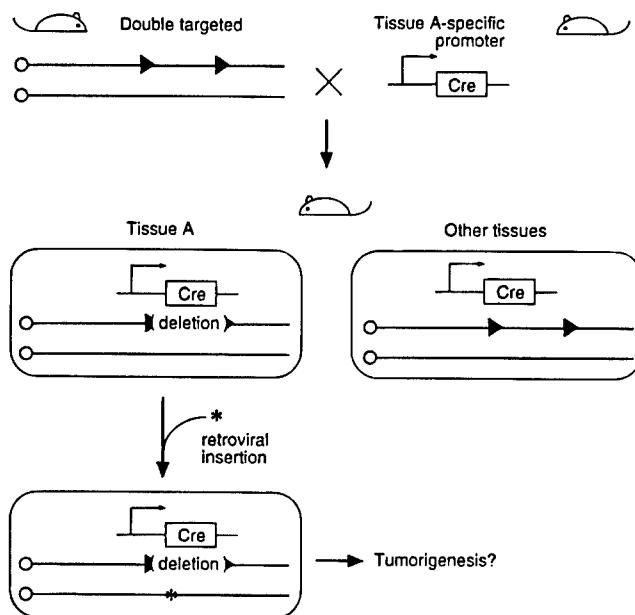

FIG. 8. Somatic chromosome engineering to model loss of heterozygosity in tumorigenesis and in screens for novel tumor suppressor genes. A double-targeted mouse line is crossed to a Cre transgenic line that expresses Cre in a tissue (or cell type, developmental stage, etc.)-specific manner, e.g., mammary specific. The progeny that inherit both the double *loxP* substrates and the Cre transgene will have deletions induced in a tissue-specific manner. A retrovirus can then be introduced to the tissue of interest to inactivate the second allele of any tumor suppressor gene within the deletion interval. If tumorigenesis occurs, the tumor tissue can be used to clone the putative tumor suppressor gene using the retrovirus tag. The retroviral insertion event may also precede the deletion event. Such an approach can also be used to simply model somatic loss of heterozygosity events by introducing a targeted mutation in a known tumor suppressor gene instead of the retroviral insertion.

cell autonomous or nonautonomous gene functions, such efficiency may be insufficient to produce a phenotype.

CONCLUDING REMARKS

The capability of ES cell technology has increased with time. We can now achieve virtually any genetic change in the mouse genome, from single-base-pair changes to large chromosomal rearrangements. Single-gene knockouts have been extremely fruitful in deciphering the functions of cloned genes and will continue to play important roles in functional studies. However, the speed at which targeted mutations are being generated lags far behind the sequence information derived from the Genome Project. Two additional approaches will become increasingly important in ascertaining functional information on a genomewide scale. Retrovirus-based gene trapping in ES cells will provide a bank of mutant ES cells for further studies in whole animals on germline transmission. Such an approach has the advantage of combining mutagenesis with gene identification or sequencing (42,43). However, the drawback of this approach is the need to transmit all the mutant alleles through germ line. In addition, the mutations generated by gene trapping represent only a limited subset of all possible mutations, with most of them being truncations. On the other hand, chemical mutagenesis with agents such as ENU has the power to provide a spectrum of different mutations, including missense and nonsense mutations and loss- and gain-of-function mutations. The availability of an allelic series for a single genetic locus will greatly help elucidate different aspects of a gene's function, for instance, functions at different developmental stages. Advances in positional cloning and functional rescue in the mouse will make identification of ENU-induced mutations an ever more efficient process. Extensive mutagenesis has not been possible for most regions of the mouse genome due to a lack of marked deletions and balancer chromosomes. The ability to generate these rearrangements at will, will prove to be invaluable for functional genomics through large-scale recessive genetic screens. Engineered rearrangements can also be used to model human conditions, as has been demonstrated in the case for the DiGeorge syndrome human (44).

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A Targeted X-Linked CMV-Cre Line

Hong Su,[1] **Alea A. Mills,**[2] **Xiaozhong Wang,**[1] **and Allan Bradley**[1,3]*
[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas
[2]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
[3]The Wellcome Trust Sanger Institute, Cambridge, United Kingdom

The Cre-*loxP* recombination system has been widely used to assess gene function in vivo, particularly when combined with established gene targeting technology. While targeted mutations and conditional alleles are routinely generated in 129 Sv/Ev ES cell lines and analyzed in 129 × B6 hybrid progeny, most *Cre* transgenic lines have been generated in a different genetic background. Therefore, experiments using Cre excision are often performed in a different genetic background than the original knockout, complicating interpretation of phenotypes. In addition, because of transgene positional effects, several *Cre* transgenic lines are usually required in order to obtain one with the desired pattern and level of Cre.

To circumvent this we generated an X-linked Cre line in the 129S5/SvEvBrd (129S5) line from which the widely used AB series of embryonic stem (ES) cells were derived and many floxed alleles are available. A Cre expression cassette under the control of the human cytomegalovirus (CMV) promoter was inserted into an insertional targeting vector designed to target the X-linked hypoxanthine phosphoribosyl transferase (*Hprt*) locus (Zhang *et al.*, 1994) (Fig. 1A). This locus was selected because mutations of *Hprt* in mice had been shown to have minimal affects (Dunnett *et al.*, 1989). Moreover, targeting the *Hprt* locus provides a convenient negative selection for accurately targeted clones. The targeting vector was linearized within the region of homology, and upon targeted insertion this will duplicate exon 2 and exon 3, mutating the *Hprt* locus. Following electroporation into AB1 ES cells, G418- and 6-TG-resistant clones were selected and targeting was confirmed by Southern analysis (Fig. 1B). The targeted allele *Hprt*$^{Tg(CMV-Cre)Brd}$ was established in mice using routine procedures.

To assess Cre activity, male *Hprt*$^{Tg(CMV-Cre)Brd}$ (CMV-Cre) mice were mated to females carrying a floxed PGK-*Neo* targeted allele. Because the Cre transgene is X-linked, all female progeny carry the transgene (inherited from their father). Cre mediated excision of the floxed PGK*Neo* was scored by PCR amplification of the tail DNA (Fig. 2A). The recombined allele generates a product that is 2.3 kb smaller than that of the nonrecombined allele because of the excision of the *PGKneo* cassette. The CMV promoter provides strong and constitutive expression in many cell types (Schmidt *et al.*, 1990). In order to determine whether the CMV-Cre line is efficient for generating excised alleles in the germ line, a male carrying both the CMV-Cre transgene and a triple-*loxP* targeted allele was mated to wild-type females (Fig. 2B; Mills *et al.*, unpublished research). All classes of recombinant alleles (Fig. 2C) were recovered in the progeny at the following frequencies: recombination between *loxP* sites 1 and 3 (5/13); recombination between *loxP* sites 2 and 3 (1/13); recombination between *loxP* sites 1 and 2 (6/13); no recombination (1/13). Independent segregation of the CMV-Cre allele and the excised alleles was also observed. Among the 12 progeny with excised alleles, six were males that do not contain the Cre allele (inherited X chromosome from their wild-type mother). These mice demonstrate that this targeted CMV-Cre transgene efficiently excises the floxed allele.

In this report, we describe an X-linked targeted Cre transgenic line in 129S5 and demonstrate that this line can be used to efficiently generate *loxP* deletions in vivo. Because this mouse line has the same genetic background as the mice derived from AB2.1 and AB2.2 ES cells (Simpson *et al.*, 1997), it should prove to be a useful addition to the available Cre lines. Finally, because the Cre transgene is X-linked, this system has an additional advantage that the Cre transgene can be simply tracked in crosses by simple observation of the sex of the progeny.

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* Correspondence to: Allan Bradley, Ph.D., The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA UK.
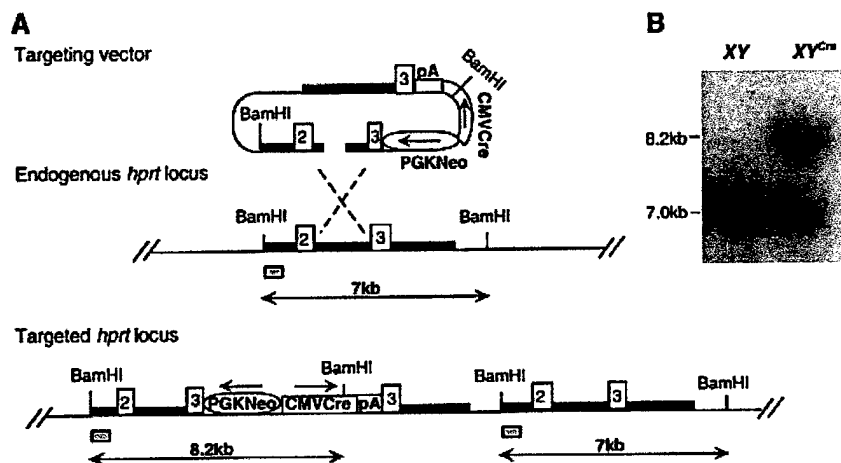E-mail: abradley@sanger.ac.uk

A

Targeting vector

Endogenous *hprt* locus

Targeted *hprt* locus

B

XY   XY^Cre

8.2kb—

7.0kb—

FIG. 1. Targeting of CMV-*Cre* into the mouse *Hprt* locus. **(A)** Schematic representation of CMV-*Cre* targeting. CMV-Cre was cloned as an *Xho*l fragment from pOG231 (O'Gorman *et al.*, 1997) into the *Xho*l site of RIV6.0 (Zhang *et al.*, 1994). The *Xho*l site is located in the third exon of the *Hprt* gene. The arrow indicates the transcription orientation. *Hprt* exons are represented by numbered boxes. **(B)** Southern analysis of targeted clones using an internal probe indicated by the gray box in **(A)**. The targeted X chromosome displays a novel 8.2-kb fragment as well as the 7-kb endogenous fragment in *Bam*HI-digested genomic DNA.

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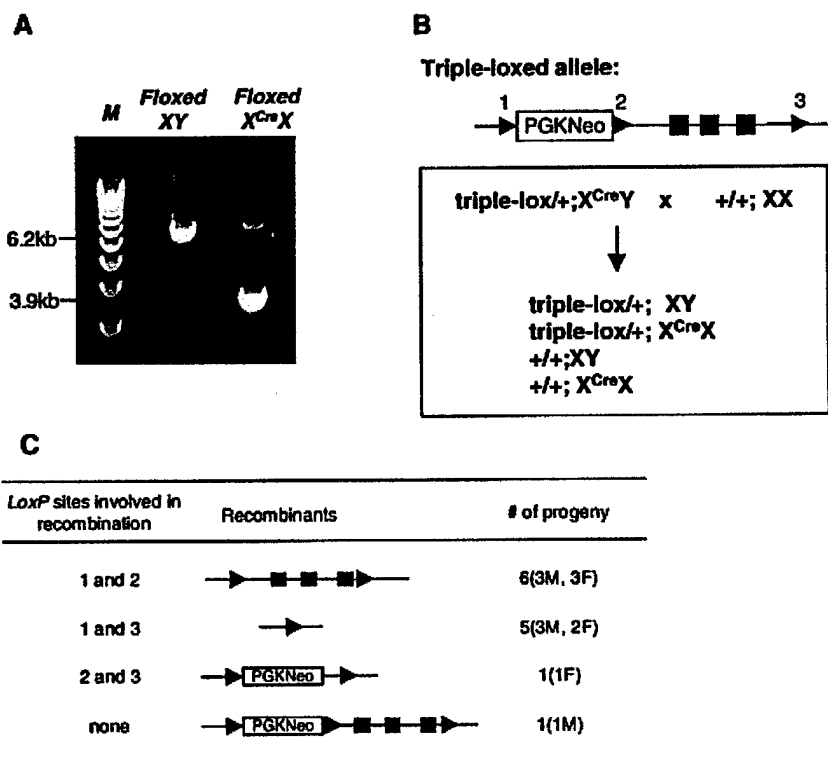
A

M   Floxed XY   Floxed X^Cre X

6.2kb—

3.9kb—

B

Triple-loxed allele:

1   2   3
PGKNeo

triple-lox/+;X^Cre Y   x   +/+; XX

↓

triple-lox/+; XY
triple-lox/+; X^Cre X
+/+;XY
+/+; X^Cre X

FIG. 2. Assessment of Cre excision activity *in vivo*. **(A)** Demonstration of Cre excision activity by polymerase chain reaction (PCR) analysis of excised alleles in pups from the mating between the CMV-*Cre* transgene and a floxed PGK-*Neo* locus. **(B)** Schematic illustration of the triple-loxed allele and the mating scheme used in the test. The *loxP* sites are represented by arrows; selection marker and exons are marked as open box and filled boxes respectively. **(C)** List of recombination products and frequency with which they were obtained from the triple-loxed allele mating. F, female; M, male.

C

LoxP sites involved in recombination	Recombinants	# of progeny
1 and 2		6(3M, 3F)
1 and 3		5(3M, 2F)
2 and 3		1(1F)
none		1(1M)